EVALUATION OF *SACCHAROMYCES CEREVISIAE* REDUCTASES AS PRACTICAL CATALYSTS FOR KETONE REDUCTIONS

By

IWONA AGATA KALUZNA

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2004

To my Family, and in a very special note to Lukasz.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Jon D. Stewart, for his guidance and encouragement, support and valuable input throughout this journey. It has been a great experience and pleasure to work with him.

I would also like to show appreciation to my Ph.D. committee for their time and contribution to this work. I would like to acknowledge Dr. James Deyrup for his care, availability, and his confidence and encouragement along these five years.

My gratitude goes to all former group members Sonia Rodriguez Giordano, Carlos Martinez, Aris Polyzos and Jennifer Tonzello for their support, and help getting me started in the group. My greater appreciation goes to Kavitha Vedha-Peters, Brian Kyte, Adam Walton and Leslie Lehman who supported and helped me move along my early years and listened to my continuous complains.

My deep recognition goes to those who spent the last years of my work with me. I would like to thank Despina "Boughkuuu" for her friendship, scientific spirit, encouragement, Greek attitude and all crazy conversations. My gratitude goes also to Brent Feske who made me realized the "importance" of the C-13 side chain of Taxol and for introducing me to the "king-cake". I would like to thank Heather Hillebrenner, Dimitri Dascier, Parag Parekh, Magda Swiderska, and Neil Stowe for their enthusiasm, and all the fun and fights we had in our lab.

I also particularly thank the undergraduate students that worked along with me on this project, Marianna Bonilla (crazy Uruguay's girl), Aileen Sewall, and Weerawut

Wittayanana (known as Tick) for their help, passion and "regenerated" results! I would like to express thanks to Tomoko Matsuda who joined me for the summer of 2002.

I want to also acknowledge my "Polish players," Gosia, Nela, and Piciek who shared the Polish strength and friendship along these years. I would like to express my gratitude to Dr. Malgosia Deyrup for her contagious enthusiasm, editorial help, and for her camaraderie.

I am greatly indebted to my family who never doubted in me and always motivated me in my study. I would not be able to accomplish anything without their endless love and support. Finally, me deepest appreciation goes to Luke who has been my fiancé, friend, soul mate and roommate in one. I thank him for his encouragement, patience, and confidence and also for his incredible help along this experience.

I hope I did not forget anyone; if I did my apologies.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

EVALUATION OF SACCHAROMYCES CEREVISIAE REDUCTASES AS PRACTICAL CATALYSTS FOR KETONE REDUCTIONS

Iwona Agata Kaluzna

August 2004

Chair: Dr. Jon D. Stewart
Major Department: Chemistry

Optically pure alcohols are valuable chiral building blocks for a variety of commercially important compounds, such as carnitine and Taxol. Baker's yeast reductions can provide inexpensive access to these materials. *Saccharomyces cerevisiae* is the most popular and well-characterized whole cell biocatalyst for asymmetric reduction of carbonyl compounds. Whole cell catalysis is convenient since it does not require an external source of cofactors; however, the yeast genome has over 40 suspected carbonyl reductases and competition among these dehydrogenases often leads to a mixture of stereoisomeric products. This problem has restricted the utility of baker's yeast for stereoselective synthesis of β-hydroxy esters and α-substituted β-hydroxy esters.

The use of purified yeast reductases avoids problems associated with competing enzymes. Identifying the substrate and stereoselectivities of the proteins is much quicker with this method as compared to previous ones. The problem lies in how to obtain the purified reductases quickly and easily. Out of almost fifty potential carbonyl reductases

present in the yeast genome, twenty-three were chosen based on sequence similarity to known reductases with broad substrate specificities. These proteins were expressed in *E. coli*, fused to a DNA sequence encoding the 26 kDa glutathione *S*-transferase protein, which serves as a tag to purify the fusion proteins. *Escherichia coli* strains overexpressing each of the twenty-three yeast reductases have been constructed and the corresponding fusion proteins have also been purified.

The construction of the library of the fusion proteins has allowed further characterization of each one of them and we have also explored applications of recombinant proteins for novel reductions. A representative set of α- and β-keto esters was tested as substrates for each purified fusion protein. The stereoselectivities of β-keto ester reductions depended both on the identity of the enzyme and the substrate structure, and some reductases yielded both L- and D-alcohols with high stereoselectivities. While α-keto esters were generally reduced with lower enantioselectivities, it was possible in all but one case to identify pairs of yeast reductases that delivered both alcohol antipodes in optically pure form. We also examined a series of α-chloro- and α-fluoro-β-keto esters as potential substrates for individual purified yeast reductases. In a majority of cases, exclusive formation of L-alcohols was observed, although formation of the opposite D-isomer was also found. This was significant because D-alcohols are produced much less commonly in biocatalytic reductions and enzymes that deliver these isomers are therefore important catalysts. Remarkably, these D-selective enzymes displayed the same stereoselectivities for both α-chloro- and α-fluoro-β-keto esters. Taken together, the results demonstrate not only that individual yeast reductases can be used to supply important chiral building blocks, but that GST-fusion proteins allow rapid identification

of synthetically useful biocatalysts (along with their corresponding genes). Furthermore, our approach is a rapid and convenient way to solve the problem of low stereoselectivity, which is often encountered when whole cell organisms are employed.

CHAPTER1
BACKGROUND AND INTRODUCTION

Homochiral α- and β-Hydroxy Esters as Building Blocks in Asymmetric Synthesis

Recently, attention has been focused on the interaction of small molecules with biological macromolecules, and the synthesis of selective enzyme inhibitors and receptor agonists or antagonists is therefore one of the key goals of the pharmaceutical industry. Understanding the interaction mechanism of biologically active molecules has led to the appreciation of the importance of chirality.

Chiral drug intermediates can be prepared by different routes. One strategy is to obtain them from naturally occurring chiral synthons, which are often produced by fermentation processes. A second approach is to carry out the resolution of racemic mixtures. This can be achieved by preferential crystallization of enantiomers or diastereoisomers and by kinetic resolution of racemic compounds by chemical or biocatalytic methods. Finally, chiral molecules can also be prepared by asymmetric synthesis using chemical strategies or biological processes based on microbial cells or isolated enzymes. The advantages of microbial or isolated enzyme- catalyzed reactions over chemical reactions are that they are often highly stereoselective and can be carried out at ambient temperature, mild pH and atmospheric pressure. These reaction conditions minimize problems of isomerization, racemization, epimerization, and rearrangement that may occur during chemical processes.

Chiral alcohols are a valuable class of chiral starting materials and a great deal of effort has been made in order to provide access to these compounds. This project was

designed to provide better stereoselectivity, higher catalytic efficiency, as well as increased diversity of available chiral starting materials by expanding the repertoire of available biocatalysts for chiral alcohol production. In recent years, it became apparent that the use of enzymes in producing important building blocks such as optically active alcohols and α- and β- hydroxy esters offers clear advantages. Research on the asymmetric reductions of halogen-containing carbonyl compounds by biocatalysis is also expanding and its practical applications to organic chemistry have resulted in success in the enantioselective synthesis of pharmaceuticals, agrochemicals and natural products.[1]

A broad collection of natural and unnatural product precursors has been synthesized from chiral alcohols and hydroxy esters in recent years.[2] One of the most studied cases is ethyl 4-chloro-3-hydroxybutanoate. The (*R*)–enantiomer (**1**) is used as a chiral building block for the synthesis of carnitine,[3,4] an essential factor for the β-oxidation of fatty acids in mitochondria and also used in the treatment of heart diseases (Figure 1- 1). The (*S*)–enantiomer (**2**) is useful as an intermediate for organic synthesis purposes.[5] Chiral diols have also been used in the synthesis of bioactive molecules. For example, all four isomers of 8-methyldec-2-yl propanoate (**3**) could be used as intermediates for the synthesis of the western corn rootworm sex pheromone.[6] Optically pure β-hydroxy esters have also found applications in the synthesis of several antibiotics (**4**),[7] and anti-asthma drugs (**5**).[8] Optically pure α-hydroxy esters are of interest as intermediates for the synthesis of a wide variety of products.[9] For instance, ethyl (*R*)-hydroxy-4-phenylbutyrate (**6**) is an essential synthon for the commercial synthesis of ACE inhibitors such as Enalapril. [10]

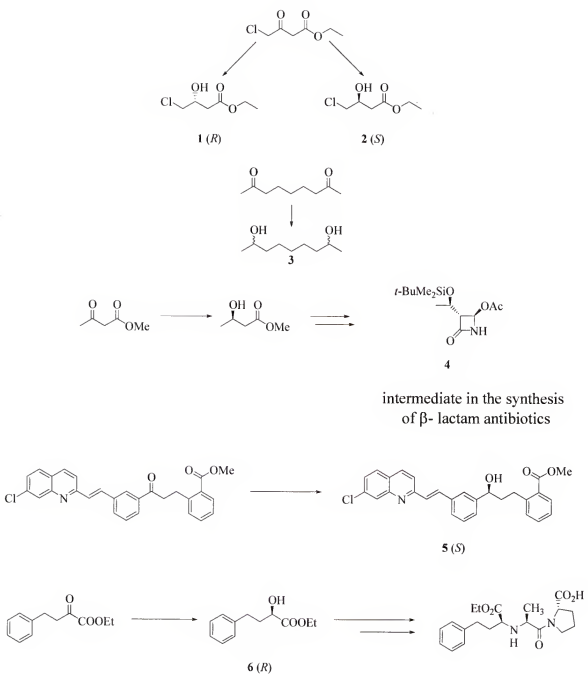


Figure 1-1. Examples of important chiral alcohols in asymmetric synthesis.

Chemical Methods Toward α- and β-Hydroxy Esters

Chemical methods for the synthesis of chiral α- and β-hydroxy esters have been widely explored. One popular technique is the asymmetric reduction of α- and β-keto esters by reagents based on sodium borohydride and lithium aluminum hydride.[11] Often

however, the required chiral auxiliaries are expensive. Potassium glucoride can be used to prepare α-hydroxy esters.[12] Unfortunately, this otherwise excellent reducing reagent is not commercially available, and its preparation is time consuming and complex.

Hydrogenation using Ru-based catalysts has been very successful for the synthesis of α- and β-hydroxy esters.[13] Unfortunately, this strategy is limited by the high cost of the catalysts. In addition, few methods are available for the synthesis of chiral α-halogeno and α-substituted β-hydroxy esters, and the diastereoselectivity of these processes is often poor. [14]

Aldol condensations [15]and stereoselective alkylations[16] of β- hydroxy esters are still used for the preparation of β-hydroxy esters, although the scope of these methods is somewhat limited.

Reductions with Metal Hydrides

Chemical and enzymatic reduction of α- and β-keto esters have been the most common approach to obtaining optically pure α- and β-hydroxy ester building blocks. Several recent reviews have described the chemical reduction of prochiral ketones including α- and β-keto esters. [2,17]

The use of metal hydride reagents for the stereoselective reduction of β-keto esters has been limited to borohydride reagents such as $LiBH_4$ and $Zn(BH_4)_2$. The stereoselectivity has been controlled by adding chiral auxiliaries,[18] or using the natural ability of β-keto esters to coordinate transition metals.[19]

Reduction with a metal hydride where the metals possess coordinating ability has been shown to induce stereoselectivity for the reduction of α-substituted β-keto ester; however, this method is only suitable for substituted β-keto esters. The

diastereoselectivity depends on the substrate and varies from 1 to 98%. Two model examples of this method have been presented in Figure 1-2.[20]

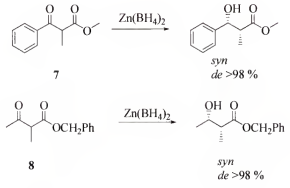


Figure 1-2. Examples of diastereoselective reduction of α-substituted β-keto esters.

Attempts to apply this method to the enantioselective reduction of non-substituted β-keto esters have been performed in presence of a bulky chiral alcohol in the ester moiety.[21]

Reduction with K-Glucoride

K-glucoride has been proven to be a highly effective chiral reagent that contains a single hydride per molecule.[12] The reagent affords high optical inductions for the reduction of prochiral ketones such as hindered aliphatic ketones, alkyl aromatic ketones, and α-keto esters. In particular, the reduction of hindered α-keto esters yields the corresponding α-hydroxy esters with high optical purities reaching 81-98 % ee (Figure 1-3). Moreover, the directions of the asymmetric reductions are consistent, with the reductions providing the corresponding alcohols enriched in the *S* enantiomers for all α-keto esters tested.

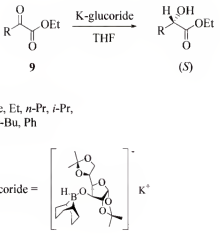
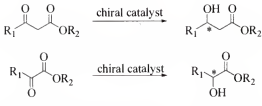


Figure 1-3. Asymmetric reductions of representative α-keto esters with K-glucoride.

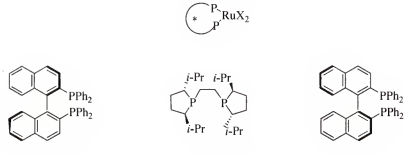
Transition Metal Catalyzed Hydrogenations

Suitable combinations of metal species and chiral organic ligands have provided some highly efficient catalysts for asymmetric hydrogenation. Electron donating ligands often increase the hydride donating ability of catalytic species. Stereo-regulation is achieved by utilizing repulsive interactions between substituents of the ligands and substrates, although bulky groups tend to decrease the reactivity. Those ligands are required in very small quantities; however, the cost associated with the use of Ru or Rh metals can make the process expensive on large scales. These types of catalysts have been widely developed and a number of applications to the reduction of α- and β-keto esters have been reported.[13,17,22]

Highly enantioselective hydrogenation of β-keto esters has been achieved with some Ru catalysts (Figure 1-4). While the BINAP-Ru dicarboxylate complexes were totally ineffective, the halogen-containing complexes RuX_2(BINAP) provided useful catalysts (Table 1-1, entries 1-4).[23] The stereoselective hydrogenation of α-keto esters was much lower compared to β-keto esters (Table 1-1, entries 14-15).[9]



Ru(II) catalysts used in hydrogenation of α- and β-keto esters



(*R*)-BINAP (*R, R*)-*i*-Pr-BPE (*S*)-BINAP

Figure 1-4. Asymmetric hydrogenation of α- and β- keto esters with Ru(II) based catalysts.

These catalysts require high temperature and pressure, and also acid co-catalysts for acceptable rates; however, milder conditions have been recently reported (Table 1-1, entries 5-7).[14] Use of bis (phospholane) ligands such as BPE also leads to highly enantioselective Ru-catalyzed hydrogenation of β-keto esters under mild conditions (Table 1-1, entries 8-10).[22,24]

Table 1-1. Asymmetric hydrogenation of β-keto esters with Ru(II) based catalysts

Entry	R_1	R_2	Catalysts	Experimental cond.	[%] yield	[%] *ee*	Conf.	Ref.
1	CH_3	CH_3	$RuCl_2$\{(*R*)-BINAP\}	30°C/ 100atm. H_2	96	99	*R*	23
2	CH_3	CH_3	$RuCl_2$\{(*S*)-BINAP\}	30°C/ 83atm. H_2	97	99	*S*	23
3	C_2H_5	CH_3	$RuBr_2$\{(*R*)-BINAP\}	30°C/ 92atm. H_2	99	100	*R*	23

Table 1-1. Continued.

4	CH_3	C_2H_5	$RuCl_2$\{(*R*)-BINAP\}	30°C/ 103atm. H_2	99	99	*R*	23
5	CH_3	CH_3	$RuBr_2$\{(*S*)-BINAP\}	R. T./ 1 atm. H_2	80	97	*S*	14
6	C_2H_5	CH_3	$RuBr_2$\{(*S*)-BINAP\}	50°C/ 1 atm. H_2	100	99	*S*	14
7	C_2H_5	CH_3	$RuBr_2$\{(*R*)-MeO-Biphep\}	50°C/ 1 atm. H_2	100	99	*R*	14
8	CH_3	CH_3	(*R, R*)-*i*-Pr-BPE-Ru	35°C/ 60 psi. H_2	-	99.3	*S*	22
9	CH_3	t-Bu	(*R, R*)-*i*-Pr-BPE-Ru	35°C/ 60 psi. H_2	-	99.4	*S*	22
10	C_2H_5	CH_3	(*R, R*)-*i*-Pr-BPE-Ru	35°C/ 60 psi. H_2	-	98.6	*S*	22
11	Cl	CH_3	(*R, R*)-*i*-Pr-BPE-Ru	35°C/ 4 atm. H_2	-	76	*R*	14
12	Cl	C_2H_5	(S)-BINAP-Ru	24°C/ 77 atm. H_2	-	56	*R*	14
13	Cl	C_2H_5	(S)-BINAP-Ru	100°C/ 100 atm. H_2	-	97	*R*	14
14	$PhCH_2CH_2$	C_2H_5	(S)-BINAP-Ru	50°C/ 20 atm. H_2	-	21	*R*	9
15	CH_3	C_2H_5	$RuCl_2$\{(*S*)-BINAP\}	50°C/ 80 atm. H_2	-	43	*S*	9

Despite the high stereoselectivities displayed by Ru (II) catalysts towards β-keto esters, few successes have been achieved for reductions of α-alkyl and α-halogeno β-keto esters, with some examples summarized in Table 1-2 and Table 1-3. It is interesting that hydrogenation of an α-chloro substrates in the presence of a BINAP-Ru(η3 – $CH_2C(CH_3)CH_2)_2$ (cod) system and (R)-MeO-BIPHEP gave exclusively the *anti* – chlorohydrins with good to high ee.[14,25] The *anti* α-chloro β-hydroxy esters were synthesized with very high diastereoselection when the β-keto esters were substituted

with linear alkyl side chain (Table 1-2, entries 1-3), although the diastereoselection was decreased with bulkier side chain (Table 1-2, entries 4-5).

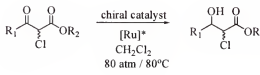


Figure 1-5. Asymmetric hydrogenation of α-chloro β- keto esters with Ru(II) based catalysts.

Table 1-2. Diastereoselectivity of Ru(II) catalysts for reduction of α-chloro β-keto esters.

Entry	R_1	R_2	Catalysts	[%] *de*	[%] *ee*	Conf.	Ref.
1	CH_3	CH_3	$RuCl_2$\{(*R*)-MeO-BIPHEP\}	99	98	(2*R*,3*R*)	14
2	CH_3	CH_3	$Ru[(\eta^3 - CH_2C(CH_3)CH_2)]_2$-(cod)-(*R*)- BINAP	98	99	(2*R*,3*R*)	14
3	C_3H_7	C_2H_5	$RuCl_2$\{(*S*)-BINAP\}	93	88	(2*S*,3*S*)	14
4	C_3H_7	C_2H_5	$RuCl_2$\{(*R*)-BINAP\}	70	90	(2*R*,3*R*)	14
5	Ph	CH_3	$RuCl_2$\{(*R*)-BINAP\}	88	99	(2*R*,3*R*)	14

High diastereoselectivity is not generally accessible in the synthesis of simple α-alkyl β-keto esters, although a handful of these reactions proceed with high level of enantioselectivity.[24,26] The degree of the efficiency of the enantio- and diastereoselection for α-alkyl and α-halogeno β-keto esters is highly influenced by substrate structure and reaction conditions.[27] The selection of solvent is especially crucial in these systems.

Ru–based catalysts generally provide a good approach for obtaining β-hydroxy esters of high optical purity; however, less progress has been made towards hydrogenation of α-alkyl and α-halogeno β-keto esters. A major problem with this approach is the use of expensive catalysts, valuable metals and chiral ligands. Attempts to

use complexes with cheaper metals, such as Co and easily available nitrogen-containing ligands have resulted in low enantiomeric excess in the conversion of β-keto esters.[28] Such problems have restricted the utility of these catalysts for practical purposes.

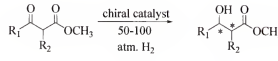


Figure 1-6. Reaction scheme for the asymmetric reduction of α-substituted β-keto esters.

Table 1-3. Diastereoselectivity of Ru(II) catalysts for reduction of α-substituted β-keto esters.

Entry	R_1	R_2	Catalyst	Solvent	*syn*:*anti*	[%] *ee*	Conf.	Ref.
1	CH_3	CH_3	(*R*, *R*)-*i*-Pr-BPE-Ru	9:1 CH_3OH/H_2O	58:42	96	2*R*, 3*R*	22
2	CH_3	NHAc	$RuBr_2$-(*R*)- BINAP	CH_2Cl_2	95:5	98	2*S*, 3*R*	23

Aldol Condensation

The aldol condensation does not provide a generally applicable route to optically pure α-alkyl β-keto esters; however, it has been successful in formation of α-methyl β-hydroxy esters. Moreover, control of enantio- and diastereoselectivity required the use of chiral enolates in stoichiometric amounts. Use of chiral reagents makes the process expensive.

In order to control both enantio- and diastereoselectivity, the use of a chiral enolate is required. A chiral ketone can be converted into each of the four possible diastereomers by regulating the stereochemistry of enolate formation and by controlling its reactivity with respect to its *si* or *re* faces. The stereochemistry of the enolates can be regulated by appropriate choice of base, whereas facial diastereoselection can be regulated by addition

appropriate choice of base, whereas facial diastereoselection can be regulated by addition of chelating or non-chelating metals. This strategy was applied using ketone (**10**) as starting material, and different combinations of base and metal resulted in formation of the four possible stereoisomers, the outcome of this approach is presented in Figure 1-7.[29,30]

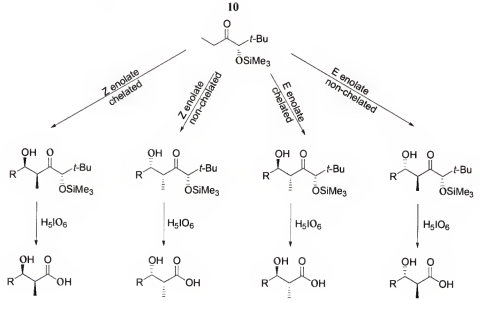


Figure 1-7. Diastereofacial selection in aldol condensations with a chiral enolate.

Alkylation of β-Hydroxy Esters

Stereoselective alkylation of optically pure β-hydroxy esters has provided these targets with good optical purity (Figure 1-8).[16] The alkylation occurs with high stereoselectivity; however the enantioselectivity is limited by the optical purity of the starting material. In these reactions, alkylation takes place on the less hindered face to yield the *anti* diastereomer. This strategy is thus limited to production of the *anti* diastereoisomer.

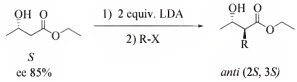


Figure 1-8. Alkylation of ethyl 3(*S*)-hydroxy butanoate

Table 1-4. Stereoselective alkylation of ethyl 3(*S*)-hydroxy butanoate.

R	[%] yield	Abs. conf.	[%] *de*
Methyl	68	2*S*, 3*S*	91
Allyl	75	2*S*, 3*S*	90
Benzyl	82	2*S*, 3*S*	94

Enzymatic Routes to Chiral Hydroxy Esters: Background

In recent years, the application of biocatalysts has become progressively more important in preparative organic chemistry. The ability of thousands of enzymes to catalyze almost every known reaction prompted the search by organic chemists for stereoselective transformations that lead to chiral intermediates in the synthesis of enantiomerically pure compounds. Enzymatic methods usually require mild conditions, which minimizes undesired side reactions. Enzymes are specific on three levels: chemo-, regio-, and stereo. Fortunately, many enzymes are not limited to their natural substrates, which dramatically expand the scope of their applicability. About 25% of the presently known enzymes are oxidoreductases,[31] among the most interesting catalysts for preparative and potential industrial applications. Dehydrogenases have therefore emerged as significant catalysts for the asymmetric production of chiral alcohols. To exhibit catalytic activity, many enzymes require a coenzyme; dehydrogenases use NADPH or

catalytic activity, many enzymes require a coenzyme; dehydrogenases use NADPH or NADH, while a few use flavin or pyrroloquinoline quinine. The reaction mechanism of the dehydrogenase reduction is as follows:

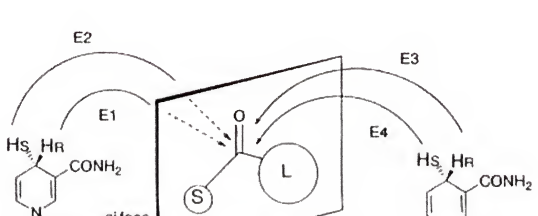


Figure 1-9. Stereochemistry of hydride transfer from NAD(P)H to the carbonyl carbon on the substrate.

There are four possible stereochemical patterns in the transfer of the hydride from the coenzyme, NAD(P)H, to the substrate (Figure 1-9). With E1 and E2 enzymes, the hydride attacks the *si*-face of the carbonyl, whereas with E3 and E4 enzymes, the hydride attacks the *re*-face. These patterns result in the formation of *R* and *S* alcohols, respectively. It has been shown that the *R*-specific system has higher affinity for substrates with large ester groups, while the keto-esters with smaller groups are preferred by the *S*-specific enzymes (Figure 1-9).[32] In practice, most alcohol dehydrogenases catalyze the transfer of the pro-*R* hydrogen of the nicotinamide to the *re*-face of a carbonyl substrate, a process predicted by Prelog's rule,[33] although dehydrogenases that lead to the formation of anti-Prelog alcohols are known.[1] Prelog's rule applies to yeast

alcohol dehydrogenase[33] and horse liver dehydrogenase. [34,35,36] The alcohol dehydrogenase from *Mucor javanicus* is specific for the pro-*S* hydrogen of NADH and *si*-face of carbonyl substrates.[37] On the other hand, the alcohol dehydrogenase from *Pseudomonas* species is specific for the pro-*R* hydrogen of NADH and *si*-face of the substrate.[38] A pro-*S*/*re*- face specific dehydrogenase has not been reported yet.

Whole Cell System

Saccharomyces cerevisiae (baker's yeast) is the most popular and well characterized whole-cell biocatalyst for asymmetric reductions of carbonyl compounds.[39,40,41] It is particularly suitable for organic synthesis since it is readily available, inexpensive, nonpathogenic, and self-contained; enzyme isolation and cofactor recycling are avoided when whole cells are used.[42] On the other hand, baker's yeast contains many kinds of dehydrogenases, some of which are *S* selective, while others are *R* selective, so that the enantioselectivities can be low to high depending on the substrate structure and the strain of baker's yeast.[43] For instance, in the reduction of 4-chloro-3-oxobutanoate (**11**) by baker's yeast, the ester moiety can be used to control the enantioselectivity of the bioconversion[44] (Figure 1-10 and Figure 1-11).

In view of the fact that baker's yeast has many different kinds of reductases, a variety of substrates can be reduced. The large array of different yeast dehydrogenases present in whole cells has made carbonyl reductions the most common application of this biocatalyst by far. Moreover, the use of whole yeast cells allows biocatalysts to be employed even when the enzyme(s) responsible for a particular conversion is not known. Some of the important and useful applications of using whole yeast cells as the biocatalyst are summarized in Figure 1-12. Reduction of ketones,[45] cyclic α-β-keto

esters,[46,47,48] diketones,[49] diketoesters[50] and fluoro-ketones[51, 52] have been reported, and in many cases the products showed high optical purity.

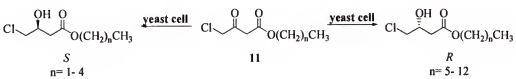


Figure 1-10. Enantioselective reduction of 4-chloro-3-oxobutanoate by baker's yeast.

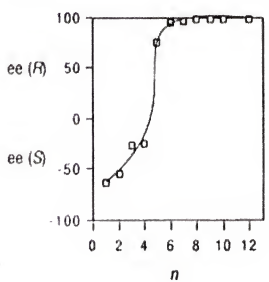


Figure 1-11. Stereochemical control on yeast catalyzed reduction by changing the ester group.[32]

While the conversions depicted in Figure 1-12 were successful, the utility of whole yeast cells is very often limited. This could be due to presence of individual enzymes with poor stereoselectivities, or to the presence of other enzymes that have the same substrate specificities but different stereoselectivities.

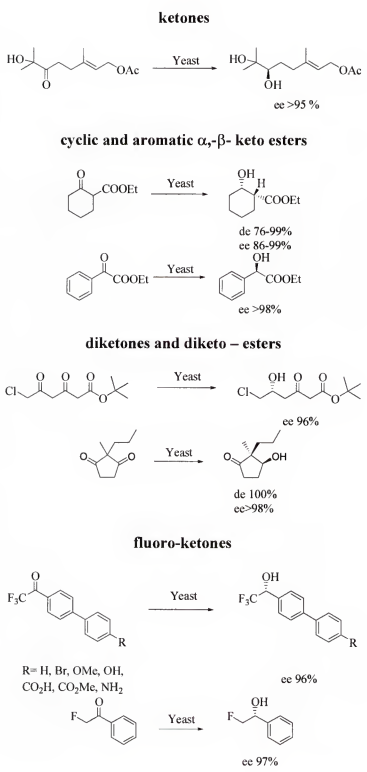


Figure 1-12. Successful applications of baker's yeast to the asymmetric reduction of carbonyl compounds.

A consensus has emerged favoring the latter notion. A variety of techniques have been used to improve the performance of baker's yeast reductions. These include changing the substrate concentration,[53,54] altering the carbon source,[55] using organic solvents or two-phase systems,[56,57] immobilizing the yeast cells,[58] adding inhibitors[59,60] or even providing sulfur compounds[61] to the reactions. The goal of all of these strategies is to decrease the catalytic activities of competing enzymes while leaving that of the target enzyme largely undisturbed. The major negative aspect of all those strategies is unpredictability of their effects on novel bio-reductions. Another inconvenience was highlighted by a report from Dao and co-workers.[62] They showed that yeast cells treated with phenacyl chloride reduced ethyl 2-oxo-4-phenyl butyrate to the corresponding *R*-alcohol with good stereoselectivity (Figure 1-13). Unfortunately, the additive significantly decreased the rate of reduction, and the high biomass/substrate ratio made this process inefficient. These negative aspects of yeast whole cells reduction made it clear to us that the use of purified enzymes might provide a better way to resolve the stereoselectivity problem in yeast reductions.

12 (*R*)
ee 80%

Figure 1-13. Enantioselective reduction by whole yeast cells.

Engineering Approach for Improvement of Whole Yeast Cell Performance

Recent developments in molecular biology have contributed to the development of useful biocatalysts.[63,64] There are several methods to improve the stereoselectivities of

carbonyl reductions by whole yeast cells. These usually involve manipulation of the activity of the enzymes directly responsible for reducing the ketone substrates. Other methods are based on manipulating metabolic pathways of yeast, to alter the redox balance and cofactor regeneration systems. Here, we have followed an alternative strategy: heterologous expression of reductases from *S. cerevisiae* in *Escherichia coli.*

We used genetic methods to build a system that combines the advantages of whole cells and isolated enzymes. The significance of the recombinant cell system can be explained by a hypothetical case: low enantioselectivity in a reduction by whole yeast cells was observed due to the presence of plural enzymes with overlapping substrate specificities but different enantioselectivities. Isolating the enzyme of interest improved the enantioselectivity; however, the requirement for enzyme purification limited the practical utility of this strategy. On the other hand, once the gene encoding the enzyme of interest was overexpressed in a host microorganism such as *E. coli*, the engineered strain behaves as an equivalent of a single enzyme within the whole cell. The fact that there is no coenzyme requirement is also a benefit for the system. The engineering approach is described in Figure 1-14.

The improvement of whole cell biotransformations by the application of genetic engineering methods has been pursued by Rodriguez et al.[88] Their first approach involved eliminating competing yeast reductases by gene knockout and overexpression of those enzymes with desirable stereoselectivity in baker's yeast.[65] Because of the limited improvements available from this strategy, reductases from baker's yeast were expressed individually in *E. coli*, and various α-keto and β-keto esters were reduced with excellent enantio- and diastereoselectivities (Figure 1-15) by whole cells of the engineered *E. coli.*

Since enzyme expression in *E. coli* is generally applicable, this strategy offered a simple way to utilize reductases from an array of different organisms. However, the problem of deciding which reductase would be most suitable remained.

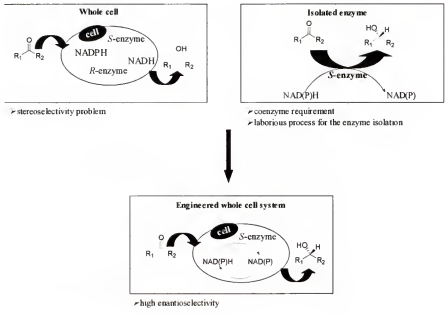


Figure 1-14. Illustration of the genetic engineering approach to improving reduction stereoselectivity.

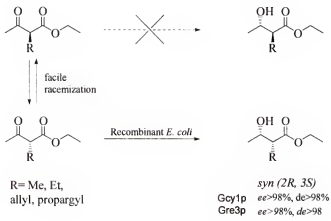


Figure 1-15. Whole cell catalyzed reduction by engineered *E. coli* cells.

Unfortunately, expression of the desired protein is always accompanied by expression of other proteins present in the host cell. The activity of the undesired proteins often interferes with catalytic activity of the overexpressed protein. Additionally, product isolation may be complicated due to large amounts of biomass and metabolites. These difficulties can be avoided using isolated enzymes.

Significance of Purified Dehydrogenases in Organic Synthesis

The most important advantages of using isolated enzymes instead of whole cell biocatalysts are their higher chemo-, regio-, and enantioselectivity caused by the absence of other competing enzymes with different stereoselectivities in the reaction system. The higher regio- and enantioselectivity of isolated yeast reductases has been widely explored by Ema et al. [66] For example, it is known that β-diketones undergo the whole cell reduction selectively at the less hindered carbonyl group. Purified reductases completely discriminate between methyl and ethyl groups as presented in Figure 1-16, structure **13**.

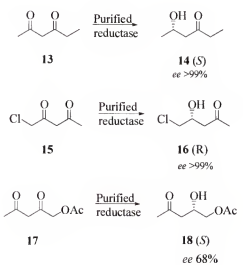


Figure 1-16. Asymmetric reduction of carbonyl compounds using isolated enzymes from *Saccharomyces cerevisiae*.

Furthermore, the regioselectivity of the reductase can be changed when the molecule is substituted with different functional groups, as in (**15**) and (**17**), indicating that the reactivity of the functional groups dominates over the steric effect [67] (Figure 1-16). The only drawback to the use of isolated enzymes is that cofactors must be provided. Both NADH and NADPH are relatively unstable molecules and expensive if used in stoichiometric amounts. Many methods have been developed for the efficient regeneration systems for these coenzymes.[68]

Yeast Dehydrogenases Involved in the Reduction of Keto Esters

Several reductases have been purified from *S. cerevisiae* based on their activity towards nonphysiological substrates.[69,70] The presence of multiple reductases active against the same substrate and the occurrence of single reductases that can accept a broad collection of carbonyl substrates has led to confusion when trying to compare results from different laboratories. In addition, several research groups have identified identical enzymes and given them different names. Sybesma[71] has reviewed the purified reductases along with their physical properties. According to Stewart et al.,[72] the yeast genome contains around 49 potential yeast dehydrogenases, which is much larger that the number reported by Sybesma.

Some of the isolated enzymes reported by Sybesma are tabulated in Table 1-5. A number of them have been shown to possess very high stereoselectivities along with broad substrate specificities. Two of the reported β-keto ester reductases[73] were named (*R*) and (*S*) according to the configuration of their products. Furuichi purified an enzyme that is responsible of the reduction of benzyl 2-methyl-3-oxobutyrate to the *syn* (2*R*, 3*S*) isomer.[74] The two D-enzymes described by Nakamura et al.[70] are similar if not identical

to those described previously by Sih et al.[44] and Heidlas et al.[73] The L-enzyme-1 and L-enzyme-2 reduced β-keto esters to corresponding L-carbinols; however, both enzymes yielded opposite diastereoisomers. [75]

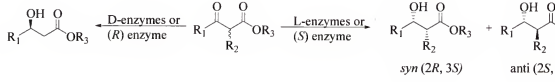


Figure 1-17. Reduction of ketones by purified enzymes.

Table 1-5. Applications of purified baker's yeast reductases

Enzyme (Name as reported in the reference)	R1	R2	R3	Absolute Conf.	% yield	% ee	% de	Ref.
L-enzyme -1	CH_3	CH_3	CH_2CH_3	2R, 3S syn	46	98	80	75
	CH_3	Allyl	CH_2CH_3	2S, 3S anti	89	98	98	
	CH_3	Propargyl	CH_2CH_3	2S, 3S anti	25	98	80	
	CH_3	Benzyl	CH_2CH_3	2S, 3S anti	<1	98	62	
L-enzyme -2	CH_3	CH_3	CH_2CH_3	2R, 3S syn	26	98	98	75
	CH_3	Allyl	CH_2CH_3	2R, 3S syn	27	98	98	
	CH_3	Propargyl	CH_2CH_3	2R, 3S syn	45	98	98	
	CH_3	Benzyl	CH_2CH_3	2R, 3S syn	4.5	98	80	
D-enzyme -1	$ClCH_2$	H	CH_2CH_3	R	62	>99	---	70
D-enzyme-2	$ClCH_2$	H	CH_2CH_3	R	80	>99	---	70

Table 1-5. Continued.

D-enzyme	$ClCH_2$	H	CH_2CH_3	R	---	>97	---	44
S-enzyme	CH_3	H	CH_2CH_3	S	---	>98	---	73
	n-propyl	H	CH_2CH_3	S	---	>98	---	
R-enzyme	CH_3	H	CH_2CH_3	R	---	>98	---	73
	n-propyl	H	CH_2CH_3	R	---	>98	---	
Fatty acid synthetase	$ClCH_2$	H	CH_2CH_3	R	---	>97	---	44
2-methyl-3-oxobutyrate reductase	CH_3	CH_3	Benzyl	2R, 3S syn	---	---	77	74

The goal of this introduction is to describe the set of potentially significant yeast reductases involved in the reduction of non-physiological ketones. This development can be done by correlating the biochemical information with the corresponding genes using the complete genome sequence of *Saccharomyces cerevisiae.*[76] The genome sequence allows one to identify all potential reductases based on sequence comparison to known reductases[77] from baker's yeast or other organisms.

Reductases are divided into families based on their size, sequence similarity and the presence of consensus motifs within the sequence. The four most studied families with relevance for carbonyl compounds reduction are the aldose reductase family, the D-hydroxy acid dehydrogenase family, the medium-chain dehydrogenase family and the short-chain dehydrogenase family (Table 1-6).[78]

Table 1-6. Overview of baker's yeast reductases.

Yeast ORF	Yeast Gene	Size [Da]	Superfamily	Known Substrates	Cofactor
YJR096w[90]	****	32,344	Aldose reductase	β-keto esters	NADPH
YDL124w[90]	****	35,560	Aldose reductase	β-keto esters	NADPH
YBR149w[83]	ARA1	38,883	Aldose reductase	D-arabinose, L-xylose, L-fructose, L-galactose, diacetyl, α-keto esters	NADPH
YOR120w[78]	GCY1	34,948	Aldose reductase	Several sugars, steroides, ketones, aldehydes, β-keto esters, diacetyl, BCO2, 6D	NADPH
YHR104w[87]	GRE3	37,014	Aldose reductase	D-xylose, β-keto esters	NADPH
YDR368w[65]	YPR1	34,755	Aldose reductase	Several sugars, steroides, ketones, aldehydes, β-keto esters, diacetyl, BCO2, 6D	NADPH
YGL185c[79]	****	43,000	D-Hydroxy acid dehydrogenase	n.d.	n.d.
YNL274c	****	38,839	D-Hydroxy acid dehydrogenase	n.d.	n.d.
YPL275w[80]	FDH2	26,487	D-Hydroxy acid dehydrogenase	n.d. suspected formate dehydrogenase activity	n.d.
YPL113c[79]	****	45,013	D-Hydroxy acid dehydrogenase	n.d.	n.d.
YOR388c[80]	FDH1	41,714	D-Hydroxy acid dehydrogenase	n.d. suspected formate dehydrogenase activity	n.d.
YLR070c[102]	XYL2	38,600	Medium-chain dehydrogenase	Xylitol, D-xylulose, β-keto esters	NADH
YMR318c[98]	ADH6	39,617	Medium-chain dehydrogenase	Several aromatic and aliphatic aldehydes and alcohols	NADPH
YCR105w[100]	ADH7	39,348	Medium-chain dehydrogenase	Several aromatic and aliphatic aldehydes and alcohols	NADPH
YAL060w[97]	BDH1	41,538	Medium-chain dehydrogenase	2,3-butanediol,	NADH
YAL061w	****	46,098	Medium-chain dehydrogenase	n.d.	n.d.

Table 1-6. Continued.

YJR159w[101]	SOR1	38,165	Medium-chain dehydrogenase	Sorbose, mannose, fructose, sorbitol,	NADH
YGL157w[106]	****	38,083	Short-chain dehydrogenase	3-oxo esters, BCO2, 6D	NADPH
YDR541c[106]	****	38,586	Short-chain dehydrogenase	3-oxo esters, BCO2, 6D	NADPH
YGL039w[106]	****	38,259	Short-chain dehydrogenase	3-oxo esters, BCO2, 6D	NADPH
YNL331c[81]	AAD14	41,991	Short-chain dehydrogenase	n.d., suspected aromatic aldehydes	n.d
YCR107w[82]	AAD3	40,911	Short-chain dehydrogenase	n.d., suspected aromatic aldehydes	n.d.
YOL151w[105]	GRE2	38,169	Short-chain dehydrogenase	Methylglyoxal, β-keto esters	NADPH

**** Protein names presented by the most common nomenclature. If the assigned gene name doesn't exist, only systematic open reading frame code identified the putative protein.
**** n.d. = not determined

Superfamilies

Aldose Reductases Family

The roles of aldo-keto reductases in many organisms, including yeast, have been difficult to define. This is partly because of overlapping substrate specificities of the family members. However, the role of some of the yeast aldo-keto reductases have been investigated and characterized. The ARA1 gene product has been identified as an arabinose dehydrogenase.[83] The GRE3 gene product has been purified and is closely related to the xylose reductase enzymes.[84] The GCY1 gene product has been recently crystallized.[85,86] The family has wide substrate specificity, including ketones, aldehydes, α- and β-keto esters[87] probably caused by an unusual flat active site.[85]

The proteins encoded by the GRE3, YPR1, and GCY1 genes play a major role in the stereoselective reduction of exogenous β-keto esters.[88,89,90] Nakamura and coworkers purified the enzyme encoded by the YBR149w gene and showed that it has the ability to

reduce a variety of α-keto esters.[91] Furthermore, in baker's yeast, these enzymes might play a role in protection against osmotic stress by producing sugar-derived polyols. Hahn-Hagerdal et al.[92] reported that Gre3p is capable of reducing xylose to xylitol in *S. cerevisiae* and that Ypr1p, Gcy1p, and Yjr096w have the ability to reduce arabinose in cell extracts. While one more yeast enzyme-Ydl124w- also appears to belong to the aldose reductase family, there is currently no information regarding the function of this protein. The amino acid sequences of all of the yeast aldose reductases are closely related to each other and to human aldose reductase, which has been characterized and crystallized.[93]

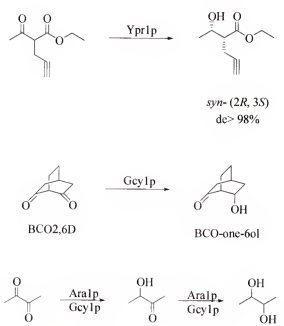


Figure 1-18. Applications of reductases from the aldose reductasefamily.

A number of key residues are conserved between the human aldose reductase and the protein sequences of the abovementioned yeast reductases. Structural information has revealed important details about the mechanism of catalysis which involves a

catalytic tetrad of amino acids (Tyr, His, Asp and Lys), where the tyrosine is the general acid/base, and the other residues appear to have auxiliary roles.[94,95] As expected, all of these residues are conserved among those yeast open reading frames.

Medium-Chain Alcohol Dehydrogenases (MDRs)

MDRs constitute a large protein family with many different catalytic abilities, including alcohol dehydrogenase, polyol dehydrogenase, glutathione-dependent formaldehyde dehydrogenase, cinnamyl alcohol dehydrogenase and quinone oxidoreductase activities.[96] Many enzymes of the MDR family have zinc in their active sites. Oxidoreductases in this family are composed of two domains, a catalytic domain and a central cofactor binding domain. The six putative yeast reductases share several key residues that are also conserved in sequence alignment of medium chain alcohol dehydrogenases.

One yeast member of the MDRs family, encoded by the YAL060w gene, has been purified and characterized.[97] The best substrates were identified as (3*R*/3*S*)-acetoin and 1-hydroxy-2-propanone. This enzyme is extremely specific for NADH as a cofactor, probably because of the glutamate residue presence in the cofactor binding site, instead of the highly conserved Asp. The YAL061w open reading frame has not yet been characterized; however, the gene shares 51% identity with YAL060w and furthermore contains a glutamate residue in the cofactor binding site, which is characteristic for NADH dependent dehydrogenases.

The likely zinc–containing alcohol deydrogenases encoded by the YMR318c and YCR105w genes are highly similar in structure and function. The sequences show a high degree of similarity (64% identity between them) and both have a serine residue in the cofactor binding site, which indicates that both are likely to be NADPH dependent

enzymes.[98] Protein products of the YMR318c[99] and YCR105w[100] genes accept a variety of aromatic and aliphatic aldehydes and alcohols. Several other members of MDR have been identified. The SOR1 codes for sorbitol dehydrogenase,[101] whereas YLR070c has been recently shown to code for xylitol dehydrogenase.[102]

Short-Chain Alcohol Dehydrogenases

The sequence of the yeast genome revealed a large number of potential proteins that might be a part of the short–chain dehydrogenase family. Proteins within this family have single-domain architecture with about 250-350 residues in the core structure that contains a conserved sequence region, which is the active site with a tetrad of catalytically important Asn, Ser, Tyr, and Lys residues of these, the Tyr is one of the most conserved residues within the whole family.[103] These dehydrogenases are metal independent enzymes that can be distinguished by a conserved Y-X-X-X-K motif.

α-Acetoxyketone reductase reduces a variety of β-keto esters to the corresponding alcohols with very high enantio- and diastereoselectivity.[104] Based on the amino acid sequence data, this enzyme was initially identified as the YJR105w open reading frame; however, Rodriguez[105] showed that this assignment was incorrect. The α-acetoxyketone reductase was purified and the amino acid sequence was determined. Data obtained by sequencing the reductase showed that the protein was actually encoded by the GRE2 gene. Based on sequence similarity to known reductases, for instance plant dihydroflavinol and cinnamoyl-CoA reductases, this gene belongs to the short-chain dehydrogenase family.

The sequence of Gre2p was used for further alignments and three closely related putative proteins were identified. Proteins encoded by the YDR541c, YGL157w, and

YGL039w genes share conserved residues between the Gre2p and plant cinnamoyl-CoA reductases.

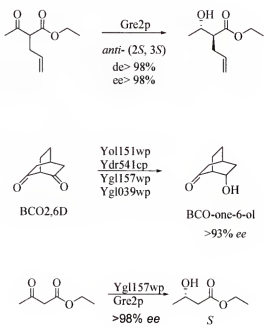


Figure 1-19. Dehydrogenases from the short chain family involved in reduction of β - keto esters.

Rodriguez reported that the protein encoded by the GRE2 gene reduces a wide range of β-keto and α-substituted β-keto esters. In addition, Katz[106] revealed that the Ygl157w and Ygl039w proteins are involved in the reduction of BCO2, 6D (bicyclo[2.2.2]octane-2,6-dione). These enzymes cover a wide range of substrates.

D-Hydroxy Acid Dehydrogenases

Enzymes from the D-hydroxyacid dehydrogenase family catalyze the stereoselective reduction of small, unbranched α-keto acids such as hydroxypyruvate and pyruvate.[107, 108] Such products have a high economic value and serve as building blocks in the synthesis of therapeutics, herbicides and insects.[109] D-lactate dehydrogenase is one

of the well known dehydrogenases, which catalyzes the reduction of pyruvate to D-lactate and also converts a wide range of 2-oxo acids to the corresponding (*R*) hydroxy acids with high enantioselectivity.[110] The substrate specificity of the D-lactate dehydrogenase is, however, substantially narrower that the L-enzyme. Moreover, the D-enzyme displayed less tolerance for side chains longer than three carbons.[111] The stereoselectivity of LDHs is very high and, apparently, the carboxylate group is involved for the correct positioning of the substrate in the active site.

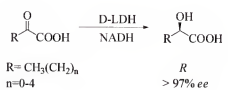


Figure 1-20. D-LDH catalyzes the reduction of α-keto acids.

NAD(P)H-dependent hydroxyacid dehydrogenases have broad substrate specificity and have been reported in many species. Analysis of the *S. cerevisiae* genome revealed five open reading frames that were similar to members of the D-hydroxyacid dehydrogenase family. Based on site-directed mutagenesis studies of the formate dehydrogense from *Pseudomonas* sp.101, three invariant residues are found in the active sites of a number of D-specific dehydrogenases: arginine, histidine and a carboxylic acid (aspartate or glutamate). A recent study confirmed that the arginine plays the key role in substrate binding and catalysis.[112] Both the arginine and histidine residues are conserved in the five open reading frames of the putative yeast D-hydroxyacid dehydrogenases. Three of them contain glutamate as the third conserved residue; however, the remaining two proteins have a glutamine instead.

Isolated Reductases versus Whole Yeast Cells System

Comparing the outcomes of stereoselective reductions using whole yeast cells with those obtained with purified yeast reductases clearly demonstrates the usefulness of individual catalysts. In nearly all cases the purified biocatalysts provided alcohol products with higher stereoselectivities. The advantages and disadvantages of using purified enzymes versus whole yeast cells are presented in Table 1-7. This evaluation below obviously displays the benefit of using purified enzymes over whole cells.

Table 1-7. Advantages and disadvantages of whole cells vs. isolated enzymes.

Parameters	Whole Yeast Cells	Isolated Reductases
Number of reductases	Many	One
Number of reactions	Many	One
Substrate and product toxicity	High	Low
Enantioselectivity	Low to High	High
Diastereoselectivity	Low to High	High
Cofactor addition	Unnecessary	Necessary
Product recovery	Difficult	Easy
Catalyst preparation	Easy	Difficult
Cost	Low	High

CHAPTER 2
A BIOCHEMICAL GENOMIC APPROACH FOR IDENTIFYING GENES FROM *SACCHAROMYCES CEREVISIAE*

Introduction

The *Saccharomyces cerevisiae* genome encodes more than 6000 proteins, each specified by an open reading frame (ORF), and forty nine of these open reading frames appear to encode potential NAD(P)H-dependent reductases.[72] This large complement makes baker's yeast one of the most useful whole cell biocatalysts for the asymmetric reduction of carbonyl compounds. Unfortunately, the presence of so many reductases with overlapping substrates specificities but differing stereoselectivities often leads to mixtures of stereoisomeric products when whole yeast cells are used for reducing exogenous substrates.

Our laboratory originally used two complementary methods to overcome the stereoselectivity problem in *S. cerevisiae*: gene overexpression and gene knockout.[65] In gene overexpression, the yeast cells were engineered to produce a large amount of a single reductase. Increasing the relative concentration of a single enzyme was expected to improve the stereoselectivity by overwhelming competing reductases. In the gene knockout approach, the absence of one or more reductases should have resulted in better stereoselectivities. Unfortunately, even when combined, these methods only partially solved the stereoselectivity problem. The yeast genome is highly redundant, and competition by additional reductases could not be easily overcome. Removal of all competing reductases using knockout methods would be time consuming and

complicated, and we therefore turned to a fundamentally different strategy based on purified yeast reductases.

While purified yeast reductases are excellent biocatalysts, their most important advantages over whole yeast cells are their higher chemo-, regio-, and enantioselectivities, since no competing enzymes with different stereoselectivities are present in the reaction mixture. On the other hand, there are also two key drawbacks with the use of isolated enzymes:

- protein isolation is often difficult, time consuming and expensive
- the reduced nicotinamide cofactor must be supplied directly.

Glutathione *S*-Transferase Fusion Proteins Systems

Martzen and co-workers[113,114] recently applied a rapid and sensitive method for isolating enzymes of interest to the yeast genome based on tag-affinity purification. Their strategy is based on the pYEX 4T-1(Clontech) expression vector, which encodes the 26 kDa glutathione *S*-transferase protein (GST)[115] from the parasite *Schistosoma japonicum* (Figure 2-1).[116] The pYEX4T vector expresses cloned genes under the control of the Cu^{+2} –inducible P_{CUP1} promoter[117,118,119] and provides a multiple cloning site for fusing the gene of interest to the *C*-terminus of glutathione *S*-transferase (GST). This permits single-step purification of fusion proteins from crude cellular lysates, since the 26 kDa protein binds reversibly and with high affinity to glutathione–containing affinity matrices.[120,121] This overcomes the problems associated with laborious protein isolation procedures.

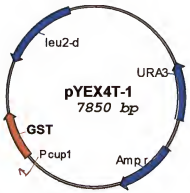


Figure 2-1. Structure of the pYEX 4T-1 vector.

***Saccharomyces cerevisiae* Overexpression System**

Knowledge of the complete genome sequence of the yeast *Saccharomyces cerevisiae* allows global approaches for the analysis of gene function. Martzen cloned each one of the 6144 open reading frames (ORFs) from baker's yeast into pYEX4T-1 (Figure 2-2).[113] A set of 6144 PCR primer pairs was constructed to amplify each of the yeast ORFs individually. All of the forward primers shared a common sequence at their 5' ends, and each of the reverse primers had a common sequence at their 5' ends. These common termini allowed re-amplification of the entire set of original PCR products using a single pair of longer primers that contained common sequences at their 3' sites. These longer flanking regions are homologous to sequences in pYEX4T-1 vector. Co-transformation of a re-PCR product with linearized pYEX4T-1 vector exploited the efficient homologous recombination machinery of yeast to insert the re-amplification product into the vector with precise fusion joints at either end. The library constructed by Martzen encompassed 6144 individual yeast strains, each expressing a different yeast open reading frame fused to glutathione *S*-transferase.

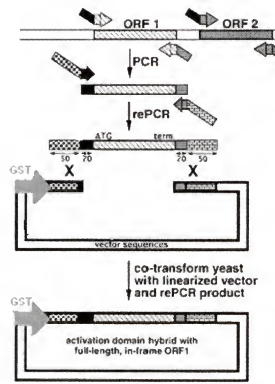


Figure 2-2. Strategy for cloning each of the yeast ORFs.

The fusion protein approach allows one to identify yeast genes responsible for specific biochemical activities, which is applicable for almost any reaction where an assay is available. This strategy has obvious applications in biocatalysis, particularly in asymmetric carbonyl reductions by yeast dehydrogenases.

While the original GST-fusion protein library included all ORFs in the *S. cerevisiae* genome, the complete collection is unnecessary for identifying dehydrogenases with desirable stereoselectivities. In an earlier analysis of the yeast genome, we identified a group of 49 ORFs likely to cover all proteins proficient in reducing carbonyl compounds of synthetic interest.[72] We further narrowed the scope of potentially useful

dehydrogenases by eliminating those known to have narrow substrate specificities or weak sequence similarities to known reductases, leaving 23 candidate ORFs (Table 2-1).

Table 2-1. Potential yeast dehydrogenases involved in carbonyl compound reductions.

Yeast ORF	Size [Da]	Yeast Gene	Superfamily
YJR096w	32,344	****	Aldose reductase
YDL124w	35,560	****	Aldose reductase
YBR149w	38,883	ARA1	Aldose reductase
YOR120w	35,079	*GCY1*	Aldose reductase
YHR104w	37,118	*GRE3*	Aldose reductase
YDR368w	34,755	*YPR1*	Aldose reductase
YGL185c	43,000	****	D-Hydroxy acid dehydrogenase
YNL274c	38,839	****	D-Hydroxy acid dehydrogenase
YPL275w	26,487	FDH2	D-Hydroxy acid dehydrogenase
YPL113c	45,013	****	D-Hydroxy acid dehydrogenase
YOR388c	41,714	FDH1	D-Hydroxy acid dehydrogenase
YLR070c	38,600	XYL2	Medium-chain dehydrogenase
YMR318c	39,617	ADH6	Medium-chain dehydrogenase
YCR105w	39,348	ADH7	Medium-chain dehydrogenase
YAL060w	41,538	BDH1	Medium-chain dehydrogenase
YAL061w	46,098	****	Medium-chain dehydrogenase
YJR159w	38,165	SOR1	Medium-chain dehydrogenase
YGL157w	38,083	****	Short-chain dehydrogenase
YDR541c	38,586	****	Short-chain dehydrogenase
YGL039w	38,259	****	Short-chain dehydrogenase
YNL331c	41,991	AAD14	Short-chain dehydrogenase
YCR107w	40,911	AAD3	Short-chain dehydrogenase
YOL151w	38,169	GRE2	Short-chain dehydrogenase
None	26,000	GST	Control

Overexpression and Purification of the GST-Yeast Reductases in Yeast

The overexpression system for each of the GST-fusion proteins generously provided by Mark Martzen utilized the pYEX 4T-1 plasmid, which was originally derived from the pYEULC vector. The plasmid is a dual-host expression vector designed for high-level expression of glutathione *S*-transferase fusion proteins in yeast. The GST-fusion protein expression is controlled by the P_{CUP1} promoter, which is induced by the presence Cu^{+2} in the growth medium. The 2μ origin of replication maintains a high plasmid copy number and the yeast selectable markers *leu2-d* (a LEU2 gene with truncated, but functional promoter) and *URA3* gene provide flexible strain selection. This plasmid also contains the ampicillin resistance gene and the pUC origin of replication for maintenance and cloning in *E.coli* (Figure 2-1).

The 23 GST-fusion proteins listed in Table 2-1 were expressed and purified according to the published procedure.[113] A description of the GST-fusion protein purification approach is presented in Figure 2-3. The yeast cells were resuspended in extraction buffer and disrupted by vortexing with glass beads (0.45-0.5mm). The residual cells and membranes components were removed by centrifugation. The supernatant was mixed with pre-equilibrated glutathione resin, and then the supernatant was discarded by decantation. The GST-fusion protein was eluted from the resin by adding free glutathione. Following dialysis, the fusion proteins were stored at -20°C.

The major reasons for using GST as a fusion partner are that many derivatives remain soluble and stable even at high levels of expression as well as the simplicity of the purification conditions. Unfortunately, it was difficult to obtain reproducible results with proteins isolated by this approach in our laboratory, mainly because of low protein yields

from the yeast overexpression systems. The yields of purified GST-fusion proteins from yeast were reported to be about 0.13-0.25mg/mL;[113] however Bradford assay and SDS-PAGE gel analyses revealed that the yields of our purified GST–fusion proteins were even lower. The SDS-PAGE gel failed to detect the expressed protein. Even though the purified proteins were isolated in low yields, they were still tested for catalytic activities.

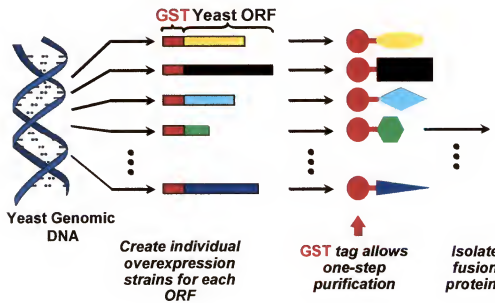


Figure 2-3. Strategy for GST-ORF fusion protein purification procedure.

Biotransformation with the Purified Yeast Dehydrogenases

Methyl acetoacetate was selected to test the catalytic activities of purified GST-fusion proteins. This β-keto ester (**19**) is known to be a good substrate for reductions by yeast whole cells.[65,87] The library of isolated yeast reductases was screened for activity toward methyl acetoacetate (Figure 2-4) at a substrate concentration of 5 mM. The glucose-6-phosphate (G6P)/glucose-6-phosphate dehydrogenase (G6PDH) system was used to regenerate a catalytic amount of NADPH in the enzymatic reductions.[122]

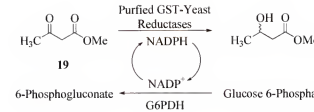


Figure 2-4. Model substrate used for testing the activity of purified GST-fusion proteins.

Each purified fusion protein reduced less than 10% of the added methyl acetoacetate after 48 hours. While it was possible that none of the 23 proteins tested accepted this substrate, we suspected that the low yields of fusion proteins were a more likely problem. It should be noted that in the examples described by Martzen et al., assays based on radioactivity were employed. The much higher sensitivities available by this technique were compatible with the low protein yields available from the yeast overexpression system. By contrast, our efforts to use yeast reductases for non-natural substrates required macroscopic enzyme quantities. We therefore explored an alternate overexpression system for the 23 GST-fusion proteins of interest.

E. coli Overexpression Systems for GST Fusion Proteins

The use of *E. coli* as a host for protein expression is convenient since many molecular biology techniques are well developed in this organism, a broad range of expression plasmids are available and growth conditions are simple and inexpensive. Moreover, based on observations made by Smith, Johnson and co-workers[123] using *E. coli* as a host and suitable prokaryotic vectors, higher expression levels of GST-fusion proteins can be reached. They also noticed that the GST-tag caused many fusions proteins to be more soluble than their native forms.

For these reasons, a new prokaryotic expression vector was constructed which incorporated the sequence encoding the GST protein, placed upstream of the polylinker region of pET26b vector (Novagen). This expression vector directed the synthesis of foreign polypeptides in *E. coli* as fusion proteins to the C-terminus of the 26-kDa glutathione *S*-transferase under control of the T7 promoter.

Construction of a Universal *E. coli* Expression Vector

The strategy used to create a plasmid for overexpressing GST-fusion proteins in *E. coli* is summarized in Figure 2-5. The glutathione *S*-transferase gene was amplified from pYEX4T-1 vector using appropriate primers that incorporated suitable restriction sites at the termini of the gene. The oligonucleotide primer for the 5' end d(ATTAATGACCAAGTTACCTATACTAGGTTAT) included an *Ase*I site (ATTAAT) adjacent to the start codon of GST. The reverse oligonucleotide sequence 5'-d(CCATGGGCATATGACGCGGAACCAGATGATCCGATT) included both *Nco*I and *Nde*I restriction sites immediately after the GST coding region. Following amplification, the GST gene was cloned into the pCR2.1-TOPO vector and sequenced. After digestion with *Nco*I and *Ase*I, the GST fragment was subcloned between the *Nde*I and *Nco*I sites of pET26b vector to yield the expression plasmid pIK2. This strategy placed two restriction sites (*Nco*I and *Nde*I) at the *C*-terminus of the GST sequence for introduction of reductase genes.

Yeast reductase genes were inserted into pIK2 by two general methods. In cases where the cloned genes were already available in our laboratory from previous studies, the appropriate DNA's were excised by restriction enzyme digestion and inserted between the *Nde*I or *Nco*I site and *BamH*I, *Hind*III, *EcoR*I, *Sal*I or *Sac*I sites (Figure 2-6).

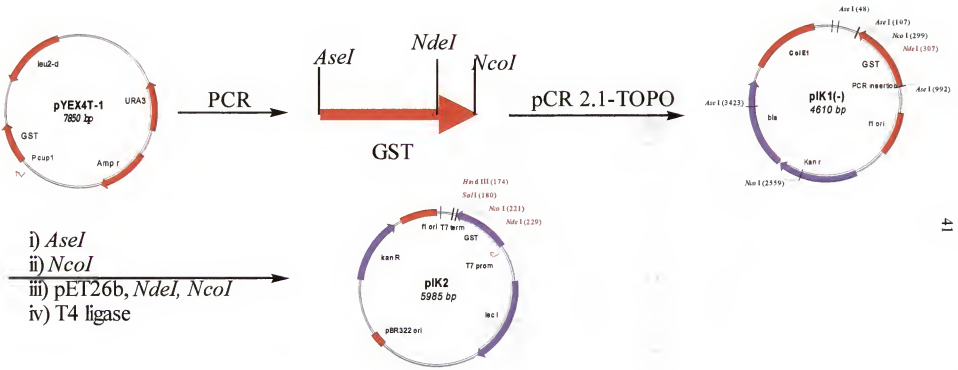


Figure 2-5. Construction of GST expression vector.

When the reductase genes were not available, they were amplified from *S. cerevisiae* genomic DNA using primer pairs that incorporated an *Nde*I or *Nco*I site at the 5'-end of the gene and a suitable restriction site at the 3'-end. After cloning into pCR2.1-TOPO vector for large-scale isolation and sequencing, the reductase genes were excised by restriction enzyme digestion and inserted into pIK2 as described in Figure 2-7.

Construction of Overexpression Systems for Aldose Reductase Family

Yeast Reductase -YJR096w

The YJR096w gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pJS375 as an *Nde*I, *Hind*III fragment and cloned between these sites in pIK2 to afford pIK9.

Yeast Reductase -YDL124w

The *E. coli* expression vector pJS374 was digested with *Nde*I and *Hind*III restriction enzymes, providing the YDL124w gene. This cassette was subcloned into the same sites in pIK2, affording pIK8 (Figure 2-6).

Yeast Reductase -YBR149w (ARA1)

The ARA1 gene was excised from pCECII as an *Nde*I, *BamH*I fragment and cloned between these sites in pIK2 to yield pIK12 (Figure 2-6).

Yeast Reductase -YOR120w (GCY1)

The GCY1 gene was PCR amplified using primers that incorporated *Nde*I and *BamH*I sites flanking the gene. After cloning the PCR product into pCR2.1-TOPO to yield pIK26(-), the insert was sequenced, and excised by digestion with *Nde*I and *BamH*I. Subcloning this fragment into the same sites of pIK2 provided pIK30. The strategy is summarized in Figure 2-7.

Yeast Reductase -YHR104w (GRE3)

The GRE3 gene was PCR amplified using primers that incorporated *Nde*I and *BamH*I sites flanking the gene. After cloning the PCR product into pCR2.1-TOPO to yield pIK27(-), the insert was sequenced, and excised by digestion with *Nde*I and *BamH*I. Subcloning this fragment into the same sites of pIK2 provided pIK29.The strategy is summarized in Figure 2-7.

Yeast Reductase -YDR368w (YPR1)

The YPR1 gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pAA1 as an *Nde*I, *Sal*I fragment and cloned between these sites in pIK2 to afford pIK4.

Construction of Overexpression Systems for D-Hydroxy Acid Dehydrogenase Family

Yeast Reductase -YGL185c

The plasmid was constructed in cooperation with Aileen K. Sewall. The YGL185c gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pJS381 as an *Nde*I, *BamH*I fragment and cloned between these sites in pIK2 to afford pAKS1.

Yeast Reductase -YNL274c

The YNL274c gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pJS390 as an *Nde*I, *BamH*I fragment and cloned between these sites in pIK2 to afford pIK13.

Yeast Reductase -YPL275w (FDH2)

The FDH2 gene was PCR amplified using primers that incorporated *Nde*I and *BamH*I sites flanking the gene. After cloning the PCR product into pCR2.1-TOPO to

provide pIK16(+), the insert was sequenced, and excised by digestion with *Nde*I and *BamH*I. Subcloning this fragment into the same sites of pET26b and pIK2 provided pIK17 and pIK18, respectively. These strategies are summarized in Figure 2-6 and Figure 2-7, respectively.

Yeast Reductase -YPL113c

The YPL113c gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pJS380 as an *Nde*I, *BamH*I fragment and cloned between these sites in pIK2 to afford pIK15.

Yeast Reductase -YOR388c (FDH1)

The FDH1 gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pCEC1 as an *Nde*I, *EcoR*I fragment and cloned between these sites in pIK2 to afford pIK14.

Construction of Overexpression Systems for Medium-Chain Dehydrogenase Family

Yeast Reductase -YLR070c (XYL2)

The XYL2 gene was PCR amplified using primers that incorporated *Nde*I and *BamH*I sites flanking the gene. After cloning the PCR product into pCR2.1-TOPO to produce pIK22(-), the insert was sequenced, and excised by digestion with *Nde*I and *BamH*I. Subcloning this fragment into the same sites of pET26b and pIK2 provided pIK24 and pIK23, respectively. These strategies are summarized in Figure 2-6 and Figure 2-7, respectively.

Yeast Reductase -YMR318c (ADH6)

The ADH6 gene was PCR amplified using primers that incorporated *Nde*I and *BamH*I sites flanking the gene. After cloning the PCR product into pCR2.1-TOPO to produce pIK19(+), the insert was sequenced, and excised by digestion with *Nde*I and

*BamH*I. Subcloning this fragment into the same sites of pIK2 provided pIK25. The strategy is summarized in Figure 2-7.

Yeast Reductase -YCR105w (ADH7)

The ADH7 gene was PCR amplified using primers that incorporated *Nde*I and *Sac*I sites flanking the gene. After cloning the PCR product into pCR2.1-TOPO to produce pIK21(+), the insert was sequenced, and excised by digestion with *Nde*I and *Sac*I. Subcloning this fragment into the same sites of pIK2 provided pIK32. The strategy is summarized in Figure 2-7.

Yeast Reductase -YAL060w (BDH1)

The plasmid was constructed in cooperation with Prof. Tomoko Matsuda. The BDH1 gene was PCR amplified using primers that incorporated *Nde*I and *BamH*I sites flanking the gene. After cloning the PCR product into pCR2.1-TOPO to yield pTM4(+), the insert was sequenced, and excised by digestion with *Nde*I and *BamH*I. Subcloning this fragment into the same sites of pET26b and pIK2 provided pTM2 and pTM3, respectively. These strategies are summarized in Figure 2-6 and Figure 2-7, respectively.

Yeast Reductase -YAL061w

The YAL061w gene was PCR amplified using primers that incorporated *Nco*I and *BamH*I sites flanking the gene. After cloning the PCR product into pCR2.1-TOPO to afford pAKS2(+), the insert was sequenced, and excised by digestion with *Nco*I and *BamH*I. Subcloning this fragment into the same sites of pIK2 provided pIK28. The strategy is summarized in Figure 2-7.

Yeast Reductase -YJR159w (SOR1)

The SOR1 gene was PCR amplified using primers that incorporated *Nde*I and *BamH*I sites flanking the gene. After cloning the PCR product into pCR2.1-TOPO to provide pIK20(+), the insert was sequenced, and excised by digestion with *Nde*I and *BamH*I. Subcloning this fragment into the same sites of pIK2 provided pIK31. The strategy is summarized in Figure 2-7.

Construction of Overexpression Systems for Short-Chain Dehydrogenase Family

Yeast Reductase -YGL157w

The YGL157w gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pJS377 as an *Nde*I, *BamH*I fragment and cloned between these sites in pIK2 to afford pIK7.

Yeast Reductase -YDR541c

The YDR541c gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pCECI as an *Nde*I, *EcoR*I fragment and cloned between these sites in pIK2 to afford pIK5.

Yeast Reductase -YGL039w

The YGL039w gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pJS376 as an *Nde*I, *Hind*III fragment and cloned between these sites in pIK2 to afford pIK6.

Yeast Reductase -YNL331c (AAD14)

The AAD14 gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pJS378 as an *Nde*I, *BamH*I fragment and cloned between these sites in pIK2 to afford pIK11.

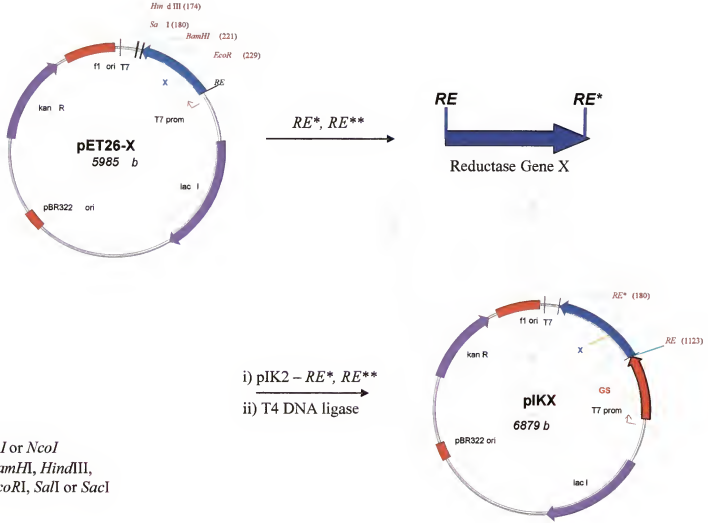


Figure 2-6. General approach for construction of *E. coli* expression vectors.

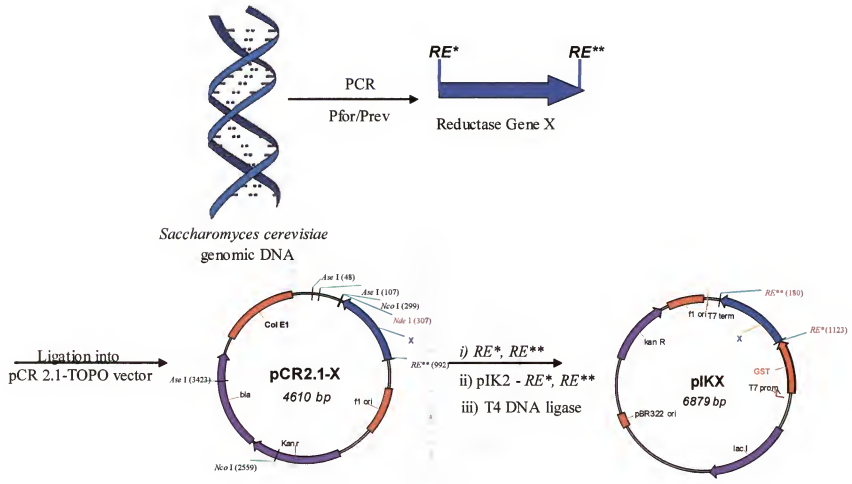


Figure 2-7. General approach for construction of *E. coli* expression vectors.

Yeast Reductase -YCR107w (AAD3)

The AAD3 gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pJS379 as an *Nde*I, *BamH*I fragment and cloned between these sites in pIK2 to afford pIK10.

Yeast Reductase -YOL151w (GRE2)

The GRE2 gene was subcloned into pIK2 vector as described in Figure 2-6. The gene was excised from pAA3 as an *Nde*I, *Hind*III fragment and cloned between these sites in pIK2 to afford pIK3.

Overexpression and Purification of the GST-Yeast Reductases in *E. coli*

After each derivative of pIK2 was constructed, it was used to transform *E. coli* strain BL21(DE3) to create the final overexpression strains. The GST-fusion proteins were induced by adding IPTG to the growth medium. In all, but four cases, fusion protein expression was successful.[124] The proteins were purified by GST-affinity chromatography as described previously, and Bradford assays showed that the concentrations of purified GST fusion reductases from *E. coli* were in the range of 0.2-3 mg/mL (data can be found in Appendix A). Figure 2-8 displays a representative SDS-PAGE gel, where YDL124w is expressed and purified as a GST-fusion protein in *E. coli*.

The results clearly show that the *E. coli* expression plasmids can produce the yeast GST-fusion proteins efficiently. This set the stage for determining the catalytic properties of each enzyme. The purified proteins and strain library are stable resources, and the purified fusion proteins have retained activity for more than a year stored -20^{0}C in 50% glycerol.

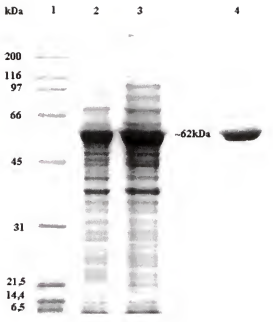


Figure 2-8. SDS-PAGE analysis showing expression and purification of GST-YDL124w. Lane 1, molecular weight marker, lane 2, cells induced with IPTG for 6hours at room temperature, lane 3, cell lysate, and lane 4, purified GST-YDL124w.

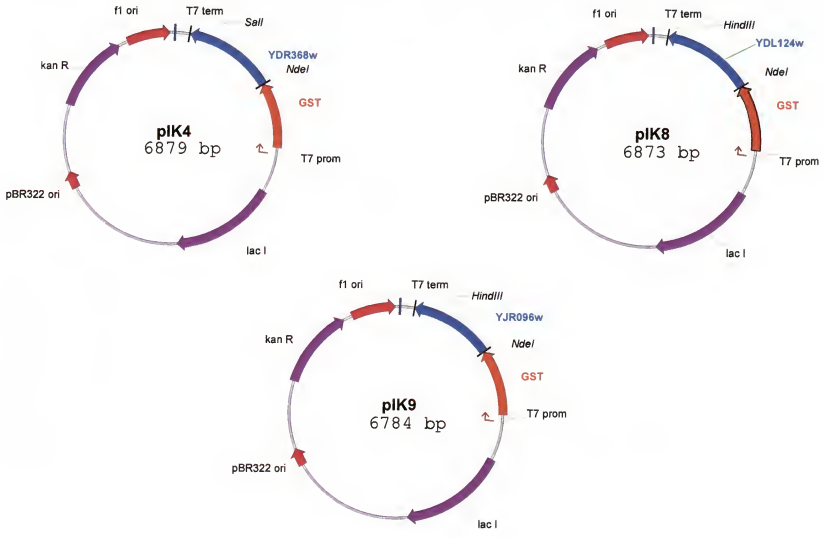


Figure 2-9. Plasmid Structures of the Aldose Reductase Family.

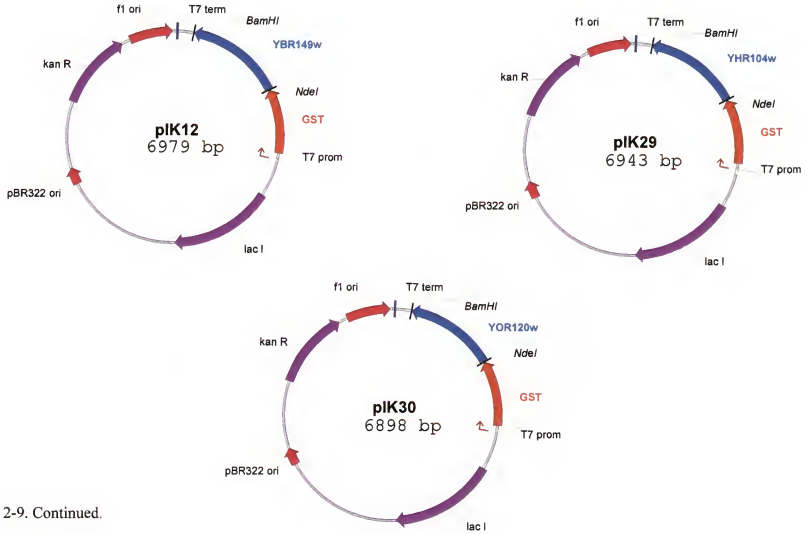


Figure 2-9. Continued.

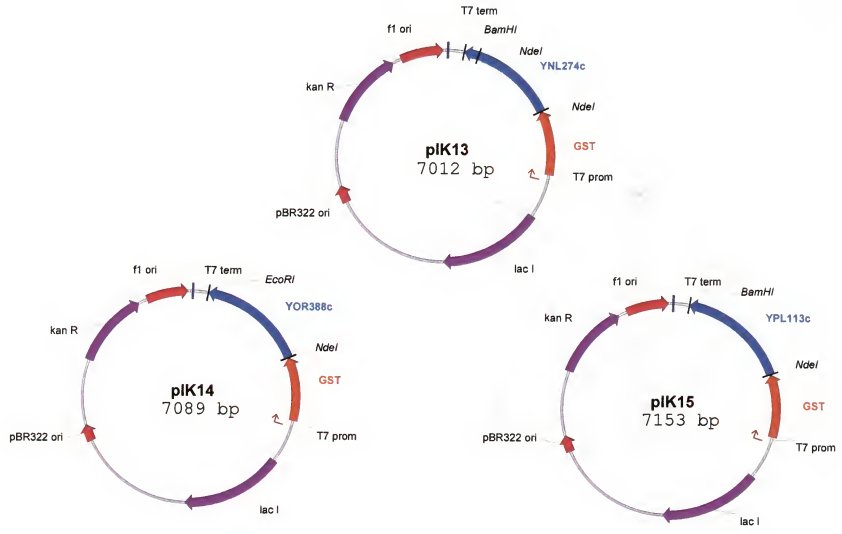
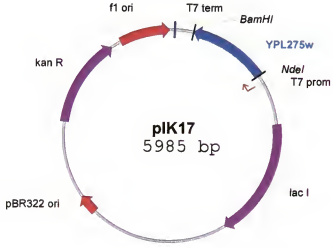
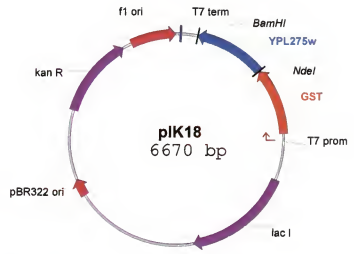


Figure 2-10. Plasmid structures of the D-Hydroxy Acid Dehydrogenase Family.





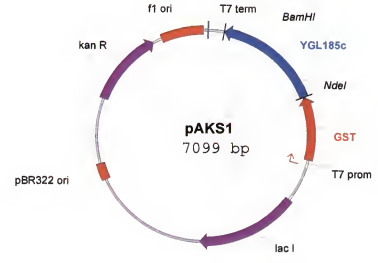


Figure 2-10. Continued.

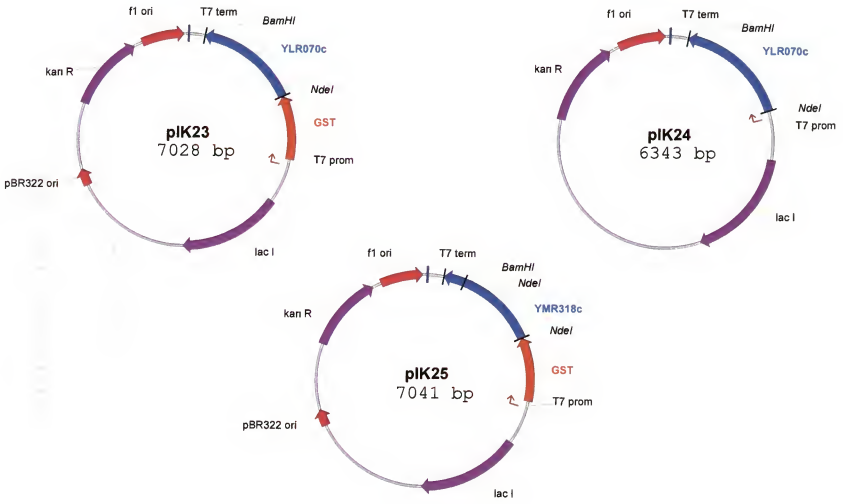
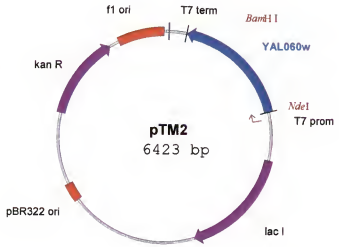
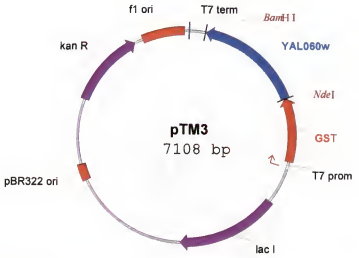


Figure 2-11. Plasmid structures of the Medium-Chain Dehydrogenase Family.





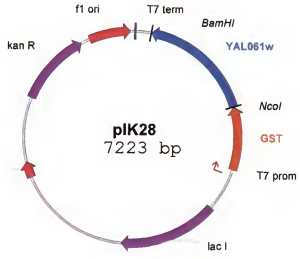
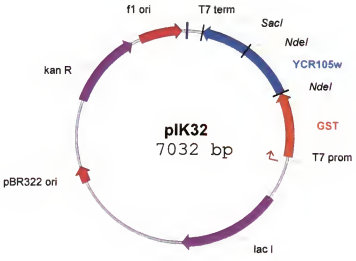


Figure 2-11.Continued.



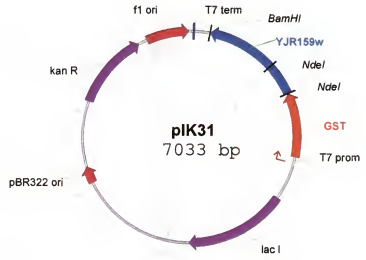


Figure 2-11. Continued.

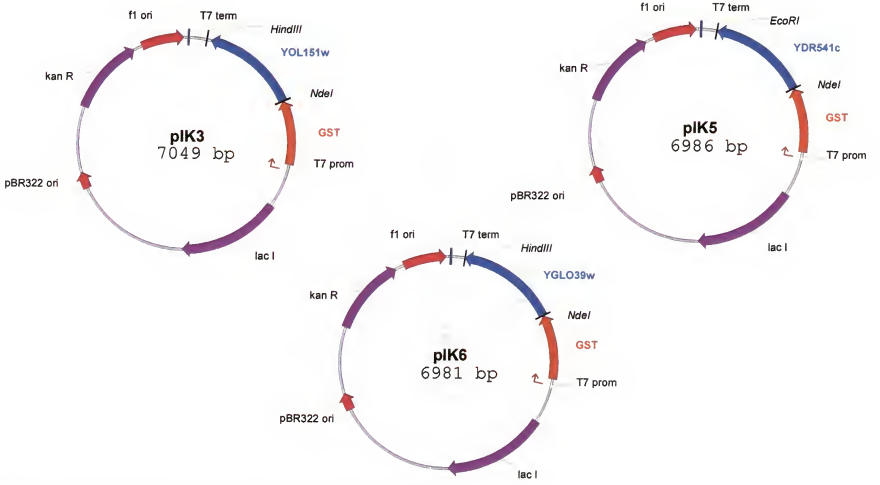
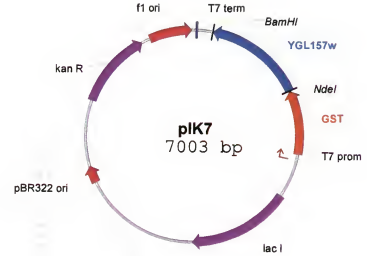
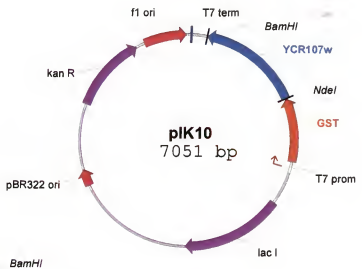


Figure 2-12. Plasmid structures of the Short-Chain Dehydrogenase Family.





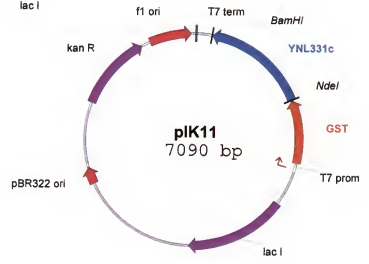


Figure 2-12. Continued.

CHAPTER 3
STEREOSELECTIVE REDUCTIONS OF α-KETO ESTERS, β-KETO ESTERS AND α-ALKYL-β-KETO ESTERS BY PURIFIED REDUCTASES FROM BAKER'S YEAST

Saccharomyces cerevisiae–Enzyme Catalyzing Carbonyl Reductions

Optically pure α- and β-hydroxy esters and α-alkyl-β-hydroxy esters provide very useful building blocks for asymmetric synthesis and a number of different methods for producing these synthons have been reported. [125,126] Asymmetric reduction of β-keto esters, either by chemical or enzymatic methods, has been a widely studied route. Chemical approaches included the use of chirally modified hydride reagents,[127] transition metal catalysts[23] and Lewis acid-mediated borohydride reductions.[128] Some of these methods are particularly important for specific classes of compounds. For example, α-unsubstituted β-hydroxy esters can be prepared via hydrogenation of corresponding β-keto esters using chiral ruthenium catalysts.[129] This method provides β-hydroxy esters in high yields and with high stereoselectivities; however, high temperatures and pressures can complicate these reactions. Moreover, chiral hydrogenations of α-substituted β-keto esters have often provided unsatisfactory results.[141] On the other hand, aldol condensations can deliver all four possible diastereomers of α-methyl-β-hydroxy esters,[29,30] although this approach cannot be generalized to other α-substituents.[130]

A number of biocatalytic regents for stereoselective β-keto ester reductions have been investigated. Among them, baker's yeast has been widely used for the synthesis of optically active alcohols because of its availability and ease of operation.[40] However, the

stereoselectivity and regioselectivity associated with the reduction of an exogenous substrate by whole yeast cell is not always satisfactory. The ideal reagent or catalyst for the production of chiral alcohols would offer broad substrate specificities, predictable access to all possible enantiomers and/or diastereomers, rapid reaction rates, low cost and would be environmentally friendly. While no system has yet been developed that meets all of these criteria, the properties of enzymes make them good candidates. This has encouraged intensive efforts to expand and improve the range and selectivities of biocatalytic carbonyl reductions. Recently, high-throughput methods have become a popular approach for evaluating "chemical" reagents and catalysts.[131,132] However, this task is usually much slower in biocatalysis because of the time required to grow microbial cells before they can be tested for their ability to carry out the desired conversion. Moreover, while "chemical" processes are typically carried out in the presence of only a single reduction catalyst or reagent, whole microbial cells contain a wealth of enzymes. When one or a few reductases with identical stereoselectivities dominate within a cell, single products can be obtained from these reactions. Often, mixtures of stereoisomeric alcohols are formed because multiple enzymes with overlapping substrate specificities but differing stereoselectivities operate concurrently. On the other hand, identification of additional microbial strains that lack the competing reductases might be the solution to this problem.[68] Unfortunately, this approach displays some disadvantages. First, it is time-consuming, and there is no simple way to direct the search rationally. In addition, a diverse range of microorganisms result from this type of screening, and each has specific nutritional and growth requirements that must be worked out individually for each case. This leads to significant increases in process development

times. Finally, even after a suitable strain has been identified, obtaining the gene encoding the key enzyme in order to produce it at higher levels in a more-easily handled host is often a time-consuming procedure, although recent progress in this area has been reported by Kim and co-workers.[133]

In our approach, we have taken an advantage of the correlation of the biochemical information to the corresponding genes using the complete genome sequence of *Saccharomyces cerevisiae.* The genome sequence allows identification of all potential reductases based on sequence comparison to known reductases from baker's yeast or other microorganisms. This strategy rapidly uncovers the most valuable enzyme(s) and provides directly the corresponding gene(s). Martzen et al. pioneered the creation of genome-wide expression libraries in which every potential open reading is expressed as a fusion protein with glutathione *S*-transferase (GST), which allows to isolate every catalyst produced by an organism by a common, one-step affinity purification (this approach was described in detail in Chapter 2).

In this chapter, the properties of twenty three *S. cerevisiae* reductases are discussed. This group was selected from our earlier analysis of the yeast genome, which revealed that 49 open reading frames encoded known or putative reductases. Approximately half of these candidates were eliminated, either because they were known or suspected to have narrow substrate specificities or their sequence similarities to known or putative reductases were low. The selection process resulted in 23 open reading frames that included members of the aldose reductase, short-chain, medium-chain and D-hydroxyacid dehydrogenase superfamilies. We considered these the most promising candidates for discovering synthetically useful biocatalysts.

Purified Yeast Dehydrogenase-Mediated Reduction of α- and β-Keto Esters

The catalytic activity of each fusion protein was assessed individually without interference from competing enzymes. Since each fusion protein is produced from the corresponding cloned gene, relationships between genotype and phenotype are established directly. In this chapter, we describe the application of the GST-methodology to the systematic study of reductases produced by baker's yeast (*Saccharomyces cerevisiae*).

Optically pure α- and β-hydroxy esters have been extensively used as chiral building blocks. The simplicity of their transformation into other useful intermediates makes them even more interesting. For example, Corrêa and co-workers have employed (*R*)-**22d** in their synthesis of (*S*)-2-methyl-4-octanol, an aggregation pheromone of *Curculionidae* species.[134] L-alcohol-product **21e** has been used to synthesize both L-carnitine[3,4] and Atorvastatin. The (*R*)-alcohol derived from α-keto ester **28c** is a key chiral building block in variety homophenylalanine-containing pharmaceuticals.

A representative group of β- and α-keto esters was selected to profile the substrate- and enantioselectivities of the isolated yeast proteins (Figure 3-1). These compounds were chosen so that to data obtained from homologous structures might reveal useful trends that could be applied to guide future synthetic applications. The entire set of α- and β-keto esters (Figure 3-1) was tested as substrates for individual cloned GST-fusion proteins.

Data for enzyme / β-keto ester pairs where only enantiomeric products are possible are shown in Table 3-1; conversions for these compounds (**20a-e**) are presented in Figure 3-3. The reductases are grouped by superfamilies, and data obtained from reactions

carried out under standard conditions with commercial yeast cells are also included for comparison.[134,135,136] It is clear from these results that all 19 of the enzymes examined in this study are indeed reductases, accepting at least one of the substrates examined. In addition, several trends can be discerned. First, there is a definite correlation between substrate size and the number of enzymes that accept simple β-keto esters: while 15 of the 19 enzymes tested catalyzed the reduction of ethyl acetoacetate **20a**, this fraction decreased to 9 of 19 for **20b** and **20c** and only 3 of 19 for **20d**. In addition, the stereoselectivities of individual reductases were not determined only by the enzyme, but depended critically on substrate structure. For example, straight-chain β-keto esters **20a-c** were all reduced primarily (often exclusively) to L-alcohols; however, branched homolog **20d** gave exclusively the D-product from the three enzymes that accepted this substrate, even though the same enzymes afforded only L-isomers for **20a-c**.

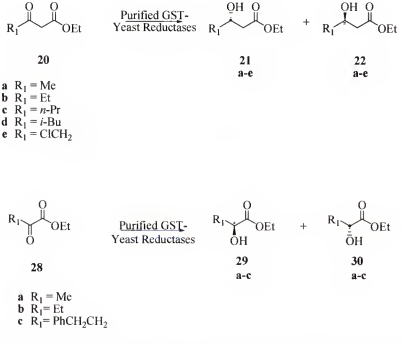


Figure 3-1. Model substrates used for the systematic study of reductases produced by baker's yeast (*Saccharomyces cerevisiae*).

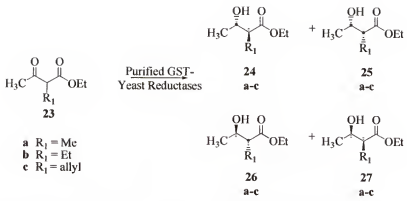


Figure 3-1. Continued.

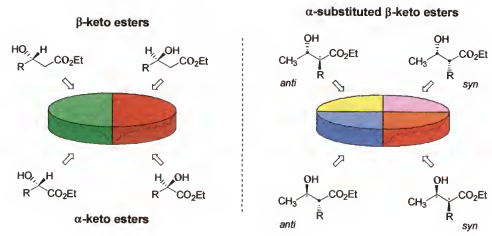
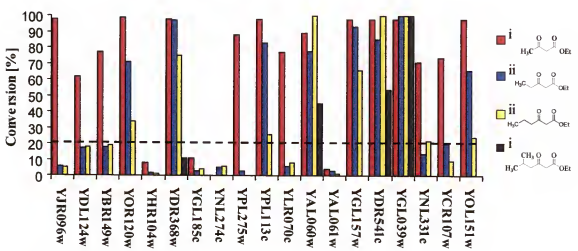


Figure 3-2. Correlations between the absolute configurations of reduction products and colors for β- and α-keto esters depicted in Tables 3-1, 3-2, and 3-3.

The enzymatic reduction of ethyl 4-chloroacetoacetate **20e** provided several interesting results. The most striking feature is that the outcomes of these reactions bore a complex relationship to those of homologs **20a-c** for all reductase superfamilies except short-chain dehydrogenases. In this latter case, reactions of **20e** afforded virtually the same stereoselectivities as **20b** and **20c**, which make similar steric demands, suggesting that substrate binding by these enzymes does not involve special interactions with the

chlorine atom. On the other hand, reductases from the other three superfamilies do not follow the same pattern. The outcomes of these reactions fall into one of two categories. For those enzymes that accept **20b** and **20c** (YOR120w, YDR368w, YPL113c and YAL060w), **20e** is reduced in a similar manner. By contrast, reductases that do not accept **20b** and **20c** (YJR096w, YDL124w, YBR149w, YHR104w, YGL185c, YNL274c, YLR070c and YAL061w) convert **20e** to primarily (or exclusively) the D-alcohol. These differing outcomes must signal differences in substrate binding orientation and underscore the difficulties in extrapolating stereoselectivity patterns to novel substrates. Indeed, it was for this reason that we selected the GST-fusion protein methodology so that empirical screening could be carried out rapidly in preference to developing active site models for each reductase.



[i] conversion after 24 hours
[ii] conversion after 72 hours

Figure 3-3. Conversion of representative β-keto esters **(20a-d).**

In Table 3-1 yeast enzymes are referred to by the systematic names of the genes encoding them. Product compositions from reactions that proceeded to ≥20% conversion within 24 hr are shown in pie charts (legend in Figure 3-2).

Table 3-1. Products from biocatalytic reductions of representative β-keto esters.

Yeast Gene					
YJR096w		---[a]	---	---	
YDL124w		---	---	---	
YBR149w		---	---	---	
YOR120w				---	
YHR104w	---	---	---	---	
YDR368w				---	
YGL185c	---	---	---	---	
YNL274c	---	---	---	---	
YPL275w		---	---	---	
YPL113c				---	
YLR070c		---	---	---	
YAL060w					
YAL061w	---	---	---	---	
YGL157w				---	
YDR541c					
YGL039w					
YNL331c		---		---	
YCR107w			---	---	

Table 3-1 Continued.

YOL151w				---	
Yeast Cells	*b*	*b*	*b*	*c*	*d*

[a]<20% conversion after 24 hours; [b]Ref. 135; [c]Ref. 134; [d]Ref. 137.

Data from reductions of representative α-keto esters using yeast GST-fusion proteins are summarized in Table 3-2, while the conversions are represented in Figure 3-4.

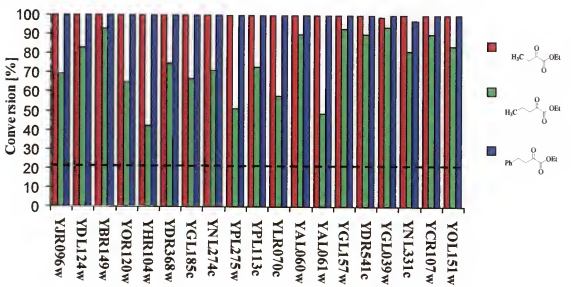


Figure 3-4. Conversions of representative α-keto esters after 24 hours **(28a-c).**

As in the previous examples, the enzymes are grouped by superfamily and the substrates are arranged in order of increasing steric bulk. Data from reductions using whole *S. cerevisiae* cells are included for comparison.[62] In line with earlier studies,[137] we have found that all three α-keto esters decomposed spontaneously at neutral pH, making it difficult to assess fractional conversion values for these reductions. On the other hand,

the alcohol products were stable to the reaction conditions, so that stereoselectivities could be determined.

In contrast to the complex results obtained from simple β-keto esters, reductions of α-keto esters generally followed similar patterns across the homologous substrate series, although there were a few exceptions. For example, aldose reductase YDR368w reduced **28a** to the corresponding L-alcohol with >98% ee; however, it displayed opposite selectivity for **28b** and **28c**. D-hydroxyacid dehydrogenase YPL275w reduced only **28a** and did not accept the larger homologs. Medium-chain dehydrogenase YLR070c presents the most puzzling case, since this enzyme reduced both **28a** and **28c**, but not **28b**. In general, α-keto esters were reduced with lower stereoselectivities than the corresponding β-keto esters, although in most cases it was still possible to identify pairs of enantiocomplementary enzymes that afforded both alcohol antipodes.

In Table 3-2 yeast enzymes are referred to by the systematic names of the genes encoding them. Product compositions from reactions that proceeded to ≥20% conversion within 24 hr are shown in pie charts (legend in Figure 3-2).

Table 3-2. Products from biocatalytic reductions of representative α-keto esters.

Yeast Gene			
YJR096w			
YDL124w			
YBR149w			
YOR120w		---[a]	
YHR104w	---	---	---
YDR368w			

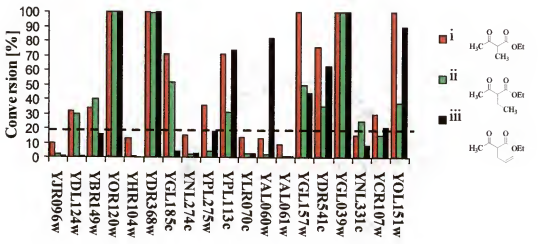
Table 3-2. Continued.

YGL185c			
YNL274c			
YPL275w		---	---
YPL113c			
YLR070c	—	---	
YAL060w			
YAL061w	---	---	---
YGL157w			
YDR541c			
YGL039w			
YNL331c			
YCR107w			
YOL151w			
Yeast Cells	 *b*	 *b*	 *c*

[a]<20% conversion after 24 hours; [b]Ref 137; [c]Ref 62.

Data for representative β-keto ester substrates that can afford diastereomeric alcohols (**23a-c**) are depicted in Table 3-3, while the conversions are represented in Figure 3-5. The high acidity of the α-protons ensures that all of these conversions are driven by dynamic kinetic resolutions. Interestingly, all of these reductions resulted in exclusive formation of L-alcohols (except for *ca.* 2% D-product formed by YAL060w from **23b**). This observation is consistent with results obtained from **20a** (Table 3-1). Three classes of behavior are evident from these results. Certain reductases–YHR104w, YGL185c, YNL274c and YAL061w–are restricted to converting only the smallest

substrate (**23a**). The remaining enzymes accept a larger variety of substrates, although they show divergent behavior with respect to stereoselectivities. For some, the preferences remain the same throughout the series of compounds. On the other hand, for five reductases, the major product from the smallest substrate is *syn*-**25a** while *anti*-**24c** dominates in the reduction of **23c**. As in the previous cases, the varied results demonstrate that directly determining reaction outcomes will likely be preferable to computer modeling strategies, at least in the near future.



[i] conversion after 72 hours
[ii] conversion after 36 hours
[iii] conversion after 24 hours

Figure 3-5. Conversions of representative α-substituted β-keto esters **(23a-c).**

In table 3-3 yeast enzymes are referred to by the systematic names of the genes encoding them. Product compositions from reactions that proceeded to ≥20% conversion within 24 hr are shown in pie charts (legend in Figure 3-2).

Table 3-3. Products from biocatalytic reductions of α-substituted β-keto esters.

Yeast Gene			
YJR096w	---[a]	---	---
YDL124w			---
YBR149w			
YOR120w			
YHR104w		---	---
YDR368w			
YGL185c		---	---
YNL274c		---	---
YPL275w		---	
YPL113c			
YLR070c		---	---
YAL060w			
YAL061w		---	---
YGL157w			
YDR541c			
YGL039w			
YNL331c			---
YCR107w		---	
YOL151w			
Yeast cells	[b]	[b]	[b]

[a]<20% conversion after 24 hours; [b]Ref. 65

Conclusions

Our data demonstrate clearly that the lack of stereoselectivity commonly observed for reductions by whole *S. cerevisiae* cells is not always due to inferior properties of the individual enzymes. This had been suspected, but therefore there had been little direct experimental data to support this contention. Many reductions with single yeast enzymes proceed with ≥90% ee and ≥90% de. The problem is that a single cell contains multiple catalysts with conflicting stereoselectivities and the net result is a mixture of products. Our genetic strategy effectively "purifies" the individual reductases so that their intrinsic properties can be discovered without interference by other enzymes. This approach also allows the properties of yeast reductases that may be poorly expressed in native cells to be assessed. It is also rapid: we routinely carry out all of the screening reactions in a total of 48 or 72 hours. These are significant advantages over earlier methods based on whole *S. cerevisiae* cells.

The current set of 19 S. *cerevisiae* reductases probably does not encompass all of the enzymes that contribute to carbonyl reductions. For example, the major product from the yeast-mediated reduction of β-keto ester **20d** is L-alcohol **21d**; however, none of the reductases surveyed in our study afforded even a trace of this enantiomer. Likewise, while D-alcohol **21c** is essentially the sole product when whole yeast cells reduce **20c**, none of the reductases examined here gave predominantly this enantiomer. Discovering the enzyme(s) with these stereoselectivities will require augmenting the current collection of GST-fusion proteins with additional yeast open reading frames.

For synthetic purposes, the ability to prepare both enantiomers of alcohol building blocks in homochiral form is essential. Hence, we designed our strategy to discover the correct enzyme pairs for each substrate rapidly, and we hoped that basing our reductase

library on the *S. cerevisiae* genome would provide sufficient genetic diversity to solve a range of synthetic problems. For α-keto esters **28a-c**, this goal has been largely realized (Table 3-2) and only L-**30b** is not available in optically pure form in this series. The situation is more complex for β-keto esters. On the one hand, both L- and D-alcohols derived from **20e** can be produced in homochiral form (Table 3-1). On the other hand, L-alcohols derived from **20a-c** and the D-alcohol from **20d** are available in >98% ee, but the antipodes are not. Likewise, only two of the four possible diastereomers derived from β-keto ester **23a** can be produced in optically pure form (*anti*-**24a** and *syn*-**25a**) and one of the four from **23b** and **23c** (*syn*-**25b, c**). Overcoming these problems will require adding additional reductases to the collection. For these purposes, the additional enzymes could be derived from *S. cerevisiae* or from any other organism whose genome has been sequenced. This ability to access potential catalysts from a diverse range of organisms that may be difficult to employ directly in bioconversions, along with the ability to discover the most appropriate biocatalyst(s) rapidly, are particularly powerful features of our fusion protein library strategy.

The other key advantage of our method is that once a suitable reductase catalyst has been identified by screening GST-fusion proteins, preparative-scale reactions can be carried out directly by whole cells of the same engineered *E. coli* strain. This provides a common platform for process development. Moreover, since each *E. coli* strain expresses only a single yeast reductase, competition by other enzymes is minimized. In this way, the benefits of rapid GST-fusion protein screening can be quickly realized in organic synthesis.

Regio- and Enantioselective Reduction of a Key β-δ-Diketo Ester

Optically active δ-hydroxy-β-keto esters are valuable chiral building blocks for the synthesis chiral drugs and natural products of polyketide origin.[138] Recently, Müller and co-workers[50] described a synthesis of the densely functionalized, enantiopure hydroxy keto ester (*S*)-**32** that relies on highly regio- and enantioselective enzymatic reduction of diketo ester **31** (Figure 3-6). While the *Lactobacillus brevis* reductase has effectively solved the problem of preparing (*S*)-**32**, the impact of this strategy would be enhanced significantly by ready access to the antipode of this key chiral building block in optically pure form. This required a reduction biocatalyst with complementary enantioselectivity.

Baker's yeast reduced diketo ester **31** at C-5 with high regioselectivity; the reduction at C-3 occurred to a negligible extent (<5%). Unfortunately, both the enantiomeric excess value and the yield of (*R*)-**33** were disappointingly low, 41% and 52% respectively. A variety of reaction parameters known to influence the performance of yeast-mediated ketone reductions were evaluated by our collaborators, Michael Wolberg and Michael Müller.

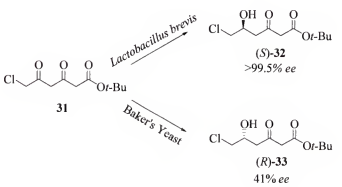


Figure 3-6. Regio- and enantioselective reduction of β-δ-diketo ester **31**.

Using resting rather than fermenting yeast cells, resulted in increased yield (72%); however, the enantiomeric excess of (*R*)-**33** increased only slightly. By contrast, a biphasic water/*n*-hexane medium significantly improved the enantiomeric purity of (*R*)-**33** to 63-78%; however the chemical yield was lower to only 38%. Neither entrapping the biocatalyst in pellets (immobilized baker's yeast), adding sucrose as a source of reducing equivalents, nor including additives improved the results of bioconversion. On the other hand, significant improvements were found by employing freeze-dried baker's yeast. This approach gave a reaction yield of 79% along with an enantiomeric excess of 69% under a single phase, aqueous conditions. Based on these results, they concluded that maximum enantioselectivity was obtained with the freeze-dried cells and since the concentration of **31** must be kept low the reaction was run in presence of an adsorber resin (Amberlite ^{TM} XAD-7).

While the process optimization studies described above provided reasonable solutions to problems of yield and stereoselectivity in the reduction of **31**, our previous results with isolated enzymes [139] suggested that these might provide even better results.

31 (*R*)-**33**

Yeast Whole Cell 94% *ee*
Purified GST-Yeast Reductases >99% *ee*

Figure 3-7. Reduction of **31** to the corresponding (*R*) alcohol.

We took an advantage of a partial library of baker's yeast ketone reductases that was assembled from an analysis of the fully sequenced yeast genome described in Chapter 2. A collection of twelve *S. cerevisiae* reductases: (YOL151w (GRE2), YDR368w (YPR1), YDR541c, YGL039w, YGL157w, YDL124w, YJR096w, YCR107w (AAD3), YNL331c (AAD14), YBR149w (ARA1), YNL274c, and YPL113c) was screened for the reduction of **31**; the results revealed that three NADPH dependent members of this library reduced diketo ester **31:** YGL157w, YOL151w, and YDR368w. All three enzymes provided the hydroxy keto ester **33** in the desired (*R*)-configuration with enantiomeric excess values of >99%, 96.5% and >99%, respectively (Figure 3-7). Enantiopure hydroxy keto ester (*R*)-**33** could be reduced on >0.5g scale this way.[140]

In conclusion, we have demonstrated that baker's yeast is a favorable biocatalyst for the synthetically valuable hydroxy keto ester (*R*)-**33** by highly regio- and enantioselective reduction of **31**. Under optimized reaction conditions involving whole cells, the product (*R*)-**33** was obtained in 94% ee and 50% isolated yield. The enantioselectivity could be improved even further by employing individual yeast reductases that were identified empirically following genome analysis. Both approaches can be applied on preparative scales.

CHAPTER 4
STEREOSELECTIVE REDUCTIONS OF α-CHLORO β-KETO ESTERS BY PURIFIED REDUCTASES FROM BAKER'S YEAST

Introduction

Enantiomerically enriched α-chloro β-hydroxy esters are useful precursors for the synthesis of optically active glycidates,[141] which are key intermediates in synthesis, for example, in commercial routes to drugs such as Diltiazem and Taxotere.[25] In addition, optically active halohydrins are useful starting materials for ferroelectric liquid crystals and pesticides.[142]

The microbiological reduction of chloro ketones by baker's yeast or other microorganisms has already been demonstrated in several examples.[143,144] Fair to good yields and high enantiomeric excess values of some respective chlorohydrins were obtained.

For example, baker's yeast has been used to reduce ethyl 2-chloro-3-oxobutanoate to a mixture of *syn*- and *anti*-chlorohydroxy esters.[145] Unfortunately, an estimation of the stereochemical purity of the products was not directly made. In a closely-related reaction, Hamdani et al.[146] have claimed the exclusive formation of the *syn*-(2*R*, 3*S*)-chloro hydroxy ester from the same substrate. A recent patent[147] has claimed the preparation of stereomeric halohydrins by reduction of the corresponding chloro oxoesters, using various microorganisms, bacteria, yeasts and fungi; however, the yields of the reduced products were generally poor, indicating undesired conversion to other products. The formation of by-products has been already reported by Azerad and co-workers.[143] When

the α-constituent is a halogen, the substrates exhibited dehalogenation. Hamdani reported the competing dehalogenation of an α-halo β-keto ester during the reduction of ethyl 2-chloro-acetoacetate.[148] Subsequent reduction of the by-product afforded the chiral dehalo β-hydroxy ester.

There still remains a need for developing efficient processes for preparing diastereo- and enantiomerically pure forms of halohydrins. Isolated yeast reductases appeared to be an obvious alternative approach for synthesizing halohydrins without problems caused by competing enzymes that usually yield dehalogenated products.

Screening GST-Fusion Yeast Reductases with α-Chloro β-Oxo Esters

In this chapter, we report the stereoselective reduction of 2-chloro-3-oxo esters by purified enzymes from baker's yeast. The resulting chloro-hydroxy esters were often obtained with excellent enantiomeric purities. A representative group of α-chloro β-keto esters was selected to outline the substrate and stereoselectivities of the isolated yeast proteins (Figure 4-1). The structures of these compounds were also selected to be analogous to the β-keto esters examined earlier. We hoped that this would allow us to define the correlation between the substrates and enzymes and also to verify whether there is any relationship between the behavior of α-alkyl and α-halo substituents in reactions with purified yeast reductases.

The required α-chloro β-keto esters were not commercially available, with the exception of ethyl 2-chloro-3-oxobutanoate. The entire group of substrates **34b-d** presented in Figure 4-1 was therefore chlorinated according to a published procedure.[149] The array of α-chlorinated substrates was tested as substrates for each of the cloned GST-fusion proteins in the presence of NADPH. Data for each enzyme / α-chloro β-keto ester

products are tabulated in Table 4-1 (substrates **35a-d**), while the conversions are presented in Figure 4-4. The reductases are organized by superfamilies, and data for reactions carried out under standard conditions with commercial yeast cells are also included for comparison.

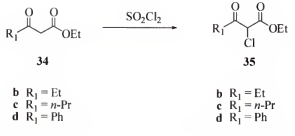


Figure 4-1. General strategy for chlorination β-keto esters.

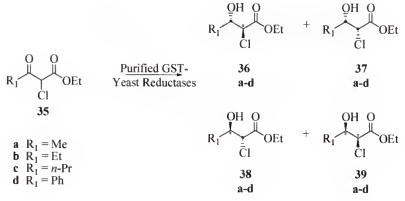


Figure 4-2. Model substrates used for the systematic study of reductases produced by baker's yeast (*Saccharomyces cerevisiae*).

In Table 4-1 yeast enzymes are referred to by the systematic names of the genes encoding them. Product compositions from reactions that exceeded to ≥20% conversion in 24 hours are shown in pie charts (legend in Figure 4-3).

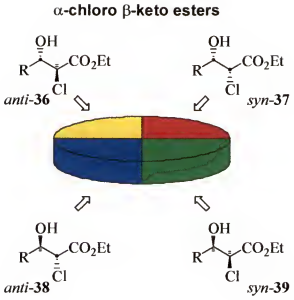


Figure 4-3. Correlations between the absolute configurations of reduction products and colors for α-chloro β-keto esters depicted in Tables 4-1.

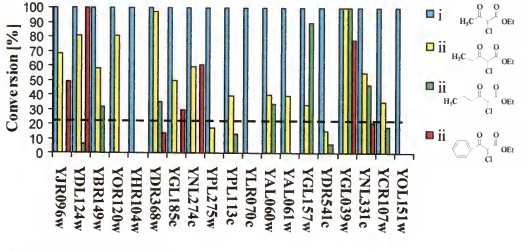
Table 4-1. Product composition from biocatalytic reductions of α-chloro β-keto esters.

Yeast gene				
YJR096w			---[a]	
YDL124w				
YBR149w				---
YOR120w			---	---
YHR104w		---	---	---
YDR368w				---
YGL185c			---	
YNL274c			---	
YPL275w		---	---	---
YPL113c				---

Table 4-1. Continued

YLR070c		---	---	---
YAL060w				---
YAL061w			---	---
YGL157w				---
YDR541c				---
YGL039w				
YNL331c				
YCR107w				---
YOL151w		---	---	---
Yeast cell	b	c	b	b

[a]<20% conversion after 24 hours; [b]Ref.143, [c] Ref. 150



[i] conversion after 24 hours
[ii] conversion after 48 hours

Figure 4-4. Conversions of representative α-chloro β-keto esters **(35a-d).**

As observed previously, there is a clear relationship between the substrate size and number of reductases that accepted the α-chloro β-keto esters. For instance, when the β-keto ester carbon chain is kept small, as in **35a,** all of the examined reductases accepted

the substrate. Increasing the carbon chain decreased the number of enzymes that accepted these substrates. In case of **35b** and **35c**, the fraction of active enzymes was 15 of 19 and 8 of 19, respectively. When the carbon chain of the β-keto ester contained a phenyl ring **(35d),** the number decreased even more drastically to only 6 reductases. This correlation is consistent with our earlier conclusions (Chapter 3). Furthermore, the stereoselectivities of YJR096w, YDL124w, YGL185c, YNL274c and YAL061w increased with increasing size of the tested compounds. These enzymes exclusively provided L-alcohols with small substrate such as **35a.** On the other hand, for larger analogs like **35c** and **35d,** formation of the opposite D-product was favored. Additionally, for phenyl-substituted **35d**, four of these purified enzymes produced only the D-alcohol **39d**. Two enzymes-YGL039w andYNL311c- afforded formation of the L-product **37d**. There are few other interesting results. Members of aldose reductase family, with the exception for YJR096w and YDL124w solely provided the L-alcohol; four enzymes gave the *syn*-**37** isomer with >95% ee and other two, YHR104 and YJR096w produced the complementary *anti*-**36** enantiomer. We also encountered this pattern with α-alkyl substituted β-keto esters. Certain reductases, like YHR104w, YPL275w, YLR070c and YAL061w were limited to accepting only the smallest substrate **35a**; on the other hand, YPL113c and YAL060w accepted almost the entire collection of α-chloro β-keto esters. These conclusions are also consistent with our findings obtained from screening the purified enzymes with α-alkyl β-keto esters. The most unusual feature is presented by YGL039w, which reduced **35a** and **35d** to the corresponding *syn*-(2*R*, 3*S*) alcohols, but converted **35b** and **35c** to the *anti*-(2*S*, 3*S*) products. In general, the enzymes from short chain dehydrogenase family

accepted a large variety of α-chloro β-keto esters, although some of them differed in their stereoselectivities.

Conclusions

The data presented in this chapter demonstrate that the use of purified enzymes can provide the desired α-chloro β-hydroxy esters in optically pure form. This approach offers clear advantages compared to other microbiological methods. For **35a,** both L-products **36a** and **37a** are available in >98% ee. Surprisingly, in the case of **35b** and **35c**, both L-alcohols **36b, c** and **37b, c** are accessible in optically pure forms. Additionally, the purified enzymes afforded formation of D-products **39b, c** in >98% ee. Both antipodes are also available in reduction of ethyl 2-chloro-3-oxophenyl propionate **35d**. The stereoselectivities of single enzymes were not only determined by the enzyme but also depended significantly on substrate structure. This is consistent with our previous outcomes with non-halogenated β-keto esters. The data obtained in these studies showed that substitution of β-keto ester in the α-position by either a methyl group or a chloro substituent furnished nearly the same results. This outcome suggested that there are no special interactions between chlorine atom and the enzymes during binding process. The remarkable result is the fact that the stereoselectivity of these enzymes is the same with the chlorine atom in 2- or 4-postion. The enzymes that accepted ethyl 4-chloro acetoacetate **20e** also accepted bulkier α-chloro β-keto esters **35b-d** and provided exclusively D-alcohols. We took advantage of these results by applying one of these reductions to total synthesis.

Synthesis of the C-13 Side Chain of Taxol

Taxol, an antimicrotubule agent isolated from the bark of *Taxus brevifolia*,[151] has recently attracted much attention because of its application in the treatment of various types of cancer. In view of promising results in cancer treatment, a large demand for Taxol has developed. The most favorable solution to the supply problem is a semi-synthetic approach involving the synthesis and attachment of (2*R*, 3*S*)-*N*-benzoyl-3-phenylisoserine to baccatin III, which can be obtained from the leaves of readily available taxus species.[152] Several syntheses of the chirally pure phenylisoserine are known,[153,154,155] but most are unsuitable for large scale production of the desired ethyl ester product.

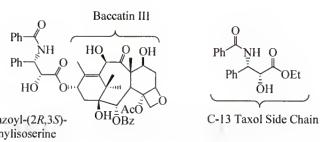


Figure 4-5. Structures of Taxol and the C-13 side chain of Taxol.

We explored a new synthetic route to obtain the desired phenylisoserine using a four step synthesis illustrated in Scheme 4-6. The key step in our approach is the stereoselective reduction of an α-chloro β-keto ester **35d** to the corresponding chlorohydrin. Azerad and co-workers have reported that ethyl 2-chloro-3-oxo-phenylpropionate was reduced in fair to good enantioselectivity by yeast or other fungal microorganisms to the corresponding 2-chloro-3-hydroxy ester stereoisomers.[143] Since

baker's yeast accepted this substrate, it was expected that one or more yeast reductases are involved in the reduction process.

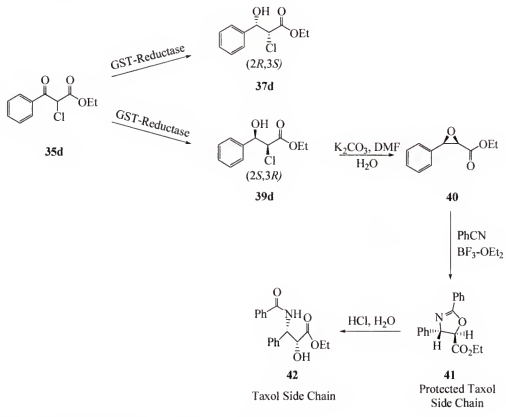


Figure 4-6. Synthesis of the C-13 side chain of Taxol.

The collection of 19 GST-yeast fusion reductases was therefore, tested for activity toward ethyl 2-chloro-3-oxo-phenylpropionate; the screening results revealed that six NADPH-dependent members of this library reduced halo ester **35d**: YGL039w, YDL124w, YJR096w, YNL331c, YNL274c, and YGL185c. Four reductases provided the desired (2*S*, 3*R*)-hydroxy ester **39d** with enantiomeric and diastereomeric excess values of >98% and >98% respectively (Table 4-2). The YGL039w and YNL331c proteins afforded the complementary (2*R*, 3*S*) isomer with high enantiomeric excess (>98%) and good diastereomeric excess values of **37d** (Table 4-2). Another student

(Brent Feske) in our group has utilized these chiral building blocks to complete the synthetic route.

Table 4-2. Reduction of ethyl 2-chloro-3-oxo-phenylpropionate by purified yeast reductases.

Superfamily	GST-Yeast Reductase	Conversion [%]	*de* [%]	*ee* [%]	Absolute conf.	
Aldose Reductase	YJR096w	49	>98	>98	(2*S*, 3*R*)	**39d**
Aldose Reductase	YDL124w	99	>98	>98	(2*S*, 3*R*)	**39d**
D-Hydroxy Acid Dehyd.	YGL185c	30	>98	>98	(2*S*, 3*R*)	**39d**
D-Hydroxy Acid Dehyd.	YNL274c	61	>98	>98	(2*S*, 3*R*)	**39d**
Short-Chain Dehyd.	YGL039w	78	80	>98	(2*R*, 3*S*)	**37d**
Short-Chain Dehyd.	YNL331c	83	78	>98	(2*R*, 3*S*)	**37d**

Substrate 35d

In conclusion, our approach clearly offers a highly practical and efficient synthesis of (2*R*, 3*S*)-*N*-benzoyl-phenylisoserine. We have shown that purified enzymes provide appropriate biocatalysts for the production of **37d** and **39d**, which are the critical chiral intermediates.

CHAPTER 5
STEREOSELECTIVE REDUCTION OF α-FLUORO β-KETO ESTERS BY PURIFIED REDUCTASES FROM BAKER'S YEAST

Introduction

α-Fluoro-β-hydroxy esters are one of the most important classes of compounds for preparing a variety of fluorine–containing molecules, particularly fluorinated counterparts of naturally occurring substances that have attracted much attention in biological and physiological chemistry.[156,157] Chiral organofluorine compounds in which a fluorine atom is bonded directly to a stereogenic center have been utilized in studies of enzyme mechanisms and as intermediates in asymmetric syntheses.[158]

Usually, the most general and reliable method for accessing α-fluoro-β-hydroxy esters is either the aldol reaction of metal enolates of monofluoroacetates[159] or the Reformatsky-type reaction of bromofluoroacetate.[160] Unfortunately, the major disadvantage of these reactions for the construction of fluoro-hydroxy esters is the modest stereoselectivities provided by these strategies. We therefore pursued the development of a stereocontrolled route to the synthesis of α-fluoro-β-hydroxy esters based on enzymatic reductions.

Based on our prior results, we expected that yeast reductases might be particularly well-suited to stereoselective reductions of α-fluoro-β-keto esters. These monofluorinated substrates are simple mimics of the unsubstituted counterparts studied earlier, and this also makes this group of compounds very interesting for us. Substitution of a hydrogen atom by fluorine in enzyme substrate analogues has been widely practiced

in various areas of bioorganic and medicinal chemistry. It is very difficult, however to generalize the relative abilities of fluorine to act as a hydrogen or hydroxy group mimic and various factors have to be considered in each circumstance. The van der Waals radius of fluorine (1.47 Å) lies between those of oxygen (1.57 Å) and hydrogen (1.2 Å) and thus fluorine appears to have a particularly close isosteric relationship to oxygen while being larger than hydrogen.[161] However, X-ray analysis reveals only small steric and geometric perturbations relative to the hydrocarbon counterparts.[162] Thus the fluorine for hydrogen substitution is not generally harmful to binding; however, the high electronegativity of fluorine can have dramatic consequences which can lead to mechanistic deviations and enzyme inhibition. [163,164,165]

Screening GST-Fusion Yeast Reductases with α-Fluoro β-Oxo Esters

The α-fluoro esters of interest were not commercially available. These compounds were therefore synthesized in three steps: α-chlorination of β-keto esters, fluorination of the remaining α-position, and finally reductive cleavage of the C-Cl bond. The synthesis was accomplished in collaboration with Weerawut Wittayanana and the collection of α-fluoro β-keto esters obtained is presented in Figure 5-1. The entire group **44a-d** was tested as substrates for each of the isolated GST-fusion yeast reductases. The screening data are presented in Table 5-1, while the conversions are tabulated in Figure 5-4. The yeast reductases are ordered by superfamilies, and also data for reactions carried out with commercial yeast cells are presented as a comparison to purified enzymes.

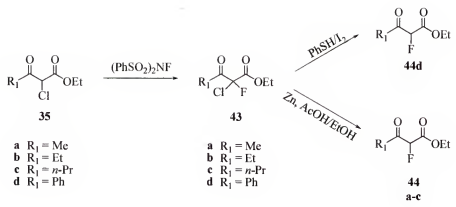


Figure 5-1. Synthesis of α-fluoro-β-hydroxy esters.

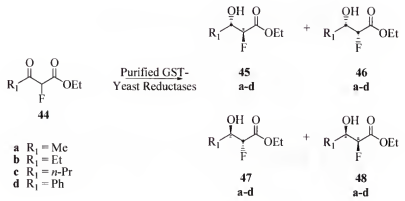


Figure 5-2. Model substrates used for the systematic study of reductases produced by baker's yeast (*Saccharomyces cerevisiae*).

The α-fluoro β-hydroxy esters obtained were characterized by ^{1}H and ^{19}F NMR, which provided the relative configurations of these molecules. The absolute configurations were assigned tentatively based on correlations to already presented outcomes in Chapter 3 and 4 since literature data were not available for these compounds. Efforts to crystallize derivatives to reveal the absolute configuration are underway.

In Table 5-1, yeast enzymes are referred to by the systematic names of the genes encoding them. Product compositions from reactions that exceeded to ≥20% conversion within 24 hr are shown in pie charts (legend in Figure 5-3).

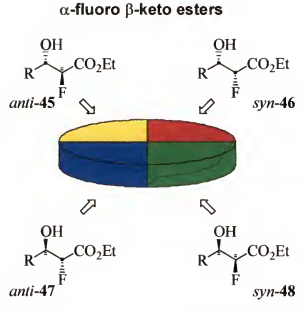


Figure 5-3. Correlations between the absolute configurations of reduction products and colors for α-fluoro β-keto esters represented in Table 5-1.

Table 5-1. Products from biocatalytic reductions of representative α-fluoro β-keto esters.

Yeast gene				
YJR096w				
YDL124w				
YBR149w				
YOR120w				
YHR104w			—	—
YDR368w				
YGL185c				

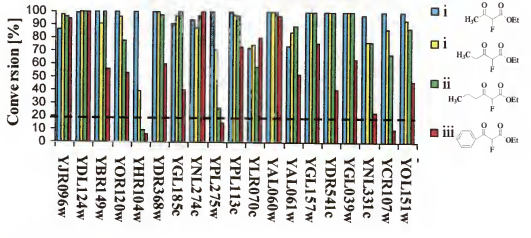
Table 5-1. Continued

YNL274c				
YPL275w				---
YPL113c				
YLR070c				
YAL060w				
YAL061w				
YGL157w				
YDR541c				
YGL039w				
YNL331c				
YCR107w				---
YOL151w				
Yeast				

[a]<20% conversion after 24 hours;

The smallest substrate, **44a,** was accepted by all of the GST-yeast reductases, although the stereoselectivity varied. Most of these isolated enzymes did not display high stereoselectivities toward this substrate, although YGL157w, YDR541c, YAL060w and YDL124w provided *syn*-**46a** and *syn*-**48a** alcohols with enantiomeric excess values over 95%. The YOR120w protein afforded mainly the opposite *anti*-**45a** product. Increasing the size of the substrate increased the stereoselectivity of individual enzymes, with a few exceptions noted for members of short chain dehydrogenase family. For example, YLR070c and YAL061w were not stereoselective toward ethyl 2-fluoro 3-oxobutyrate

44a. On the other hand, with substrates possessing one or two additional carbon atoms in the position, the stereoselectivity increased significantly. The stereoselectivities of other enzymes- YJR096w, YDL124w, YGL185c, YNL274c, YLR070c, and YAL061w-were also improved ee values >95% when the size of the substrate increased (molecules **44b-d**) and the major products in all cases were D-alcohols. This behavior is important since D-stereoselective enzymes are not that common among yeast reductases.



[i] conversion after 24 hours
[ii] conversion after 48 hours
[iii] conversion after 72 hours

Figure 5-4. Conversions of representative α-fluoro β-keto esters (44a-d).

The YAL060w member of medium chain dehydrogenase family was highly stereoselective toward the entire spectrum of fluorinated substrates (**44a-d**), exclusively affording formation of *syn*-**46a-d** products with ee >98%, whereas two other members of this family favored production of the complementary *syn*-**48** enantiomer for substrates **44b-d**. Few members of short chain dehydrogenase family displayed high

stereoselectivity for **44b-c**. These primarily yield **the** *syn*-**48** alcohols, but unfortunately these enzymes were not selective toward the phenyl substituted substrate **44d.**

In general, purified yeast reductases converted small α-fluoro β-keto ester such as **44a** primarily to L-alcohol (except for YDL124w); conversely for substrates **44b-d**, the outcomes of these biotransformations were very diverse. Thirteen of these enzymes afforded L-alcohols, but there was also a number of enzymes that produced solely D-alcohols with ee >98%. The pattern of D-selective enzymes was consistent with results obtained from screening these enzymes with α-chloro β-keto esters.

Conclusion

In general, the entire group of α-fluoro β-keto esters was accepted by reductases from our GST-library. The stereoselectivities were generally lower compared to results obtained from screening these enzymes with α-alkyl and α-chloro-β-keto esters. Surprisingly, YPL275w and YLR070c accepted almost the entire set of substrates **44a-d**. Based on previous results with α-alkyl and α-chloro or even with unsubstituted β-keto esters, we expected that these enzymes would only have accepted small substrates. In fact, while these enzymes showed little stereoselectivity toward small substrate such as **44a,** the outcomes were improved by increasing the size of the substrates **44b-d**.

We were surprised to discover that a number of enzymes delivered D-alcohols from the fluorinated β-keto esters. This was a relatively rare occurrence in other reductions by yeast enzymes.

In most cases, tested α-fluoro-β-keto esters favored formation of *syn*-alcohols, with one exception for YOR120w.

We noticed that the α-fluoro esters (**44a-d**) were much more soluble in aqueous solutions than their hydro- and chloro-counterparts. O'Hagan et al.[162] also reported increase of solubility after an F for H replacement.

Use of purified enzymes allowed us to prepare two of the four possible diastereoisomers in optically pure form in every case, expect for the small substrate **44a.** The replacement of the α-proton with fluorine atom probably did not change the steric or binding interactions of these molecules with our enzymes; however the outcome of these results was different compared to α-alkyl or α-chloro substituted β-keto esters.

CHAPTER 6
CONCLUSIONS AND FUTURE WORK

This work has clearly demonstrated that the lack of stereoselectivity commonly observed for reductions by whole yeast cells is not always due to poor properties of the individual enzymes. This has been suspected, but there had not been sufficient experimental data to support this contention. Our results confirmed this statement since many of our reductions carried out by single/purified enzymes proceed with much higher enantio- and diastereoselective values compared to results obtained from whole yeast cells-mediated reductions. This finding proved that *S. cerevisiae* genome contains a large number of "players" involved in the reduction of keto esters. Furthermore, these reductases display similar substrate specificities, but they differ in their stereoselectivities and the result is a mixture of stereoisomeric products. We solved the stereoselectivity problem by isolating these major participants. Our genetic strategy allowed us to clone and effectively purify the reductases of interest. Each reductase could then be profiled individually without interference by other competing enzymes. Twenty-three yeast reductases, major contributors in the reduction of α- and β-keto esters, were isolated and partially characterized. Unfortunately, only nineteen of these twenty-three reductases were expressed in sufficient amount in *E. coli* cells.

The fact that we had a library of nineteen powerful catalysts "in hand" permitted us to profile not only the enzymes but also variety of substrates. A number of α- and β-keto esters were screened as substrates for these purified enzymes. Many important chiral α-

and β-hydroxy esters were prepared. For the α-keto esters tested, it was possible to obtain both enantiomers in optically pure forms in most cases. In the case of unsubstituted β-keto esters only the L-enantiomer was available. Likewise, only two of the four possible diastereoisomers can be obtained from α-substituted β-keto esters. We also discovered that ethyl 4-chloro-3-hydroxy butanoate can be reduced by almost every reductase from our collection. According to previous reports, only four yeast reductases accept this substrate; we proved that the statement was incorrect.

A series of α-chloro- and α-fluoro-β-keto esters were tested as substrates for each purified reductase. In a majority of cases, exclusive formation of L-alcohols was observed, although formation of the opposite D-isomer was also found. This was significant because D-alcohols are produced much less commonly in biocatalytic reductions and enzymes that deliver these isomers are therefore important catalysts. Remarkably, these D-selective enzymes displayed the same stereoselectivities for both α-chloro- and α-fluoro-β-keto esters. In general, the purified yeast reductases showed enhanced stereoselectivities toward bulkier substrates with two or more carbon atoms in a β-chain.

Using our approach, we can obtain important chiral building blocks for organic synthesis. For example, we have produced homochiral ethyl (*2S, 3R*)-2-chloro-3-hydroxy-3-phenylpropionate, which is the key intermediate in a novel synthesis of the Taxol C-13 side chain. The (*R*)-alcohol derived from α-keto ester **28c** is a key synthon in commercial synthesis of Enalapril.[10] Both antipodes of ethyl 4-chloro-3-hydroxy butanoate are important: the (*R*)-enantiomer is used as a chiral building block for the

synthesis of carnitine [3,4] and the complementary (*S*)-isomer is a significant intermediate for organic synthesis purposes. [5]

This approach afforded much better results than those obtained with earlier techniques and also it was easier to identify the appropriate biocatalysts. Use of this strategy can help to explore applications for other optically pure hydroxy esters to the synthesis of drugs or natural products such as those described in the introduction. The other key advantage of our approach is that once a suitable reductase has been identified by screening fusion proteins, preparative-scale reactions can be carried out directly by whole cells of the same engineered *E. coli* strain.[166] Moreover, since each *E. coli* strain expresses only a single yeast reductase, competition by other enzymes is limited. In this way, the benefits of rapid GST-fusion protein screening can be quickly realized in organic synthesis.

There are number of diastereoisomers that are produced by whole yeast cells but were not detected in our conversions. Overcoming these problems will require adding additional reductases to the collection. For instance, the YMR226c protein was recently reported as member of the short-chain dehydrogenase family and displayed high activity toward stereoselective reduction of bicyclic diketones.[167] For these reasons, additional enzymes could be derived form baker's yeast or from any other organism whose genome has been sequenced.

Finally, in order to profile these enzymes more fully, a number of additional substrates need to be screened. Moreover, our yeast reductases were different in their stereoselectivities and specificities toward α-fluoro-β-keto esters when compared to the non-fluorinated analogues. This result brought our attention since monofluorinated β-keto

esters are simple mimics of the unsubstituted counterparts. Additional studies are required in order to interpret or even predict the influence of fluorine on the stereochemical outcome of given enzyme reactions after an F for H replacement.

CHAPTER 7
EXPERIMENTAL

Biochemicals and Media

Bacto-Tryptone, Bacto-Yeast Extract, Bacto Peptone and Bacto Nitrogen Base without amino acids were purchased form Difco. Dextrose was obtained from Fisher. Kanamacyin was purchased from Sigma. Amino acids used as supplements in the media were purchased from Sigma.

Restriction endonucleases were purchased from New England Biolabs or Promega. T4 DNA ligase was obtained from New England Biolabs. Oligonucleotides were obtained from Integrated DNA technology or Geno Mechanix. *Taq* polymerase and *Pfu* polymerase were purchased from New England Biolabs.

Vectors

E. coli expression vectors pET 22b(+) and pET 26b(+) and *E. coli* cloning vectors pCR2.1-Topo and pCRTOPO II-BLUNT were obtained from Novagen and Invitrogen, respectively. Yeast expression vector pYEX 4T-1 was a generous gift from Dr. M. Martzen.

E. coli strains

E. coli strains XL1-blue, JM109, TOP10 were used for routine cloning procedures while BL21(DE3) was used for protein expression.

Table 7-1. *E coli* strains used in this work and their genotype.

E. coli strain	Genotype
XL1-blue	F'::Tn*10 proA*$^+$ *B*$^+$ *lacI*^{q} *Δ(lacZ)M15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* (r_K^- m_K^+) *supE44 relA1 lac*
JM109	F' *traD36 lacI*^{q} *Δ(lacZ)M15 proA*$^+$ *B*$^+$/ e14$^-$(McrA$^-$) *Δ(lac-proAB) thi gyrA96* (Nal^r) *recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 relA1*
TOP10	F$^-$ *mcr*A Δ(*mrr –hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G
BL21(DE3)	F$^-$ *ompT [lon] hsd*S_B (r_B^- m_B^-; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene.

***S. cerevisiae* strain**

The yeast strain used for fusion protein expression was EJ 758 [MATa *his*3-Δ200, *leu*2-3,112, *ura*3-52, *pep4::HIS3*]; a derivative of JHRY-20-2Ca was obtained from Dr. M. Martzen.

Maintenance of *E. coli* and *S. cerevisiae* strains

Standard media (LB) and techniques for routine growth and maintenance of *E. coli* strains were used.[168] Recipe per liter: Bacto-Tryptone (10g), Yeast Extract (5g), NaCl (10g). For solid media, agar (15g/L) was included. When appropriate, ampicillin was added to 200 μg/mL or kanamacyin was used at concentration of 40 μg/mL.

Non-selective yeast growth medium (YPD) was used for routine growth and maintenance of *S. cerevisiae* strains. Recipe per liter: Bacto-Yeast Extract (10g), Bacto-Peptone (20g), and dextrose (20g).[169] Agar (20g/L) was included for preparation of solid media. Selective media was used for routine maintenance of recombinant strains. Recipe per liter: Bacto Yeast Nitrogen Base without amino acids (6.7g), dextrose (20g), and the appropriate amino acids or uracil according to the autotrophic marker present in strain. L-

tryptophan (20 mg/L), L-histidine (20 mg/L), L-leucine (30 mg/L), L-methionine (20 mg/L), and uracil (2 g/L). For solid media, agar was added (20 g/L).

For short term storage, yeast and bacterial strains were kept on plates sealed with Parafilm at 4°C. For long term storage strains were stored at -80°C in media containing 20% (v/v) glycerol.

Recombinant DNA Technologies

Recombinant DNA procedures were carried out essentially as described by Sambrook et al.[168] *Saccharomyces cerevisiae* DNA used as template in the PCR reactions was isolated following the medium scale protocol reported by Wach et al.[170] Genes were amplified using a Perkin-Elmer Geneamp PCR system 2400, purified by low melt agarose gel electrophoresis and digested with appropriate restriction enzymes for cloning into desired vectors. Restriction enzyme digestions were checked by DNA gel electrophoresis. DNA agarose gel electrophoresis was carried out as described by Sambrook.[168] *E. coli* transformations were performed by electroporation.[168] Mini-preparations of plasmid DNA were performed using alkali lysis, phenol extraction and EtOH or *i*-PrOH precipitation. Purified DNA for sequencing was obtained by density gradient ultracentrifugation with CsCl in presence of ethidium bromide.

DNA sequencing was done using the Big Dye Terminator reactions and chromatography of samples was run at the DNA Sequencing Core Facility of the University of Florida.

Table 7-2. Sequences of primers used for amplifying genes needed for this study. Restriction enzymes recognition sites are underlined.

Gene	Primer set
GST	For 5'-ATTAATGACCAAGTTACCTATACTAGGTTAT-3' Rev 5'-CCATGGGCATATGACGCGGAACCAGATGATCCGATTT-3'

Table 7-2 Continued.

YOR120w	For 5'-CATATGCCTGCTACTTTACATGATTCTAC-3' Rev 5'-GGATCCTTACTTGAATACTTCGAAAGGAGACC-3'
YHR104w	For 5-CATATGTCTTCACTGGTTACTCTTAATAAC-3' Rev 5'-GGATCCTCAGGCAAAAGTGGCGAATTTACCA-3'
YPL275w	For 5'-CATATGGTGGTCATCAATAAGCAATTAA-3' Rev 5'-GGATCCTTATTTCTTCTGTCCATAAGCTCTGGT-3'
YLR070c	For 5'-CATATGACTGACTTAACTACACAAGAAGCT-3' Rev 5'-GGATCCCTTGGATGCCAAAAGTTA-3'
YMR318c	For 5'-CATATGGTCTTATCCTGAGAAATTTGAAGG-3' Rev 5'-GGATCCTAGTCTGAAAATTCTTTGTCGTAGC-3'
YCR105w	For 5'-CATATGCTTTACCCAGAAAAATTTCAGGG-3' Rev 5'-GAGCTCTATTTATGGAATTTCTTATCATAATCGA-3'
YAL060w	For 5'-CATATGAGAGCTTTGGCATATTTCAAGAAG-3' Rev 5'-GGATCCTTACTTCATTTCACCGTGATTGTTAGG-3'
YAL061w	For 5'-CCATGGATGAGAGCCTTAGCGTATTTCGGTAA-3' Rev 5'-GGATCCTCATGTGTGACGCAGTTTAGCCTC-3'
YJR159w	For 5'-CATATGTCTCAAAATAGTAACCCTGCAGTA-3' Rev 5'-GGATCCTCATTCAGGACCAAAGATAATAGTCTT-3'

Expression and Isolation of Yeast GST-Fusion Proteins

***E. coli* system**

A fresh plate was streaked from the appropriate frozen stock and a single colony was used to inoculate 10 mL culture of the overexpression strain in LB medium containing 40 μg/mL kanamycin. After shaking overnight at 37°C, the culture was diluted 1: 100 into 500 mL of the same medium in a 2 L baffled flask. The culture was shaken at 37°C until the optical density at 600 nm reached 0.5-1.0, then isopropylthio-β-D-galactoside was added to a final concentration of 100 μM and the culture was shaken for an additional 6 hours at room temperature. The cells were collected by centrifugation,

washed twice with cold sterile water, and then resuspended in 25 mL of 100 mM KP_i, pH 7.0. All purification steps were carried out at 0 – 4°C. The cells were lysed by passage through a French pressure cell and debris was removed by centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was mixed with an equal volume of 50 mM Tris-Cl, 4 mM $MgCl_2$, 1 mM DTT, 10% glycerol, pH 7.5 and loaded onto a 2.4 × 5.0 cm column of glutathione resin (Clontech) at a flow rate of 0.5 mL/min. that had been equilibrated with 50 mM Tris-Cl, 4 mM $MgCl_2$, 1 mM DTT, 500 mM NaCl, 10% glycerol, pH 7.5 (wash buffer). The flow-through was discarded and the resin was washed twice with 20 mL wash buffer. Material eluted by wash buffer was also discarded. Essentially pure GST-fusion proteins were eluted with 40 mL of freshly prepared elution buffer (wash buffer (39.6 mL) plus 2 M NaOH (0.40 mL) and solid glutathione (0.31 g)). The eluant was dialyzed against 20 mM Tris-Cl, 2 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT, 55 mM NaCl, 50% glycerol, pH 7.5 prior to storage at -20°C.

***S. cerevisiae* system**

A 5 mL culture of the of the appropriate overexpression strain was grown overnight at 30°C in SD medium supplemented with leucine (30mg/mL), then 1 mL of the pre-culture was diluted in 50 mL SD media. This culture was grown overnight at 30°C, then it was added to 250 mL of SD medium. This final culture was shaken at 30°C until the optical density at 600 nm reached 0.80. Copper sulfate was added to a final concentration 0.50 mM, then the culture was shaken for 2 hours at 30°C. The cells were collected by centrifugation, washed twice with cold sterile water, and then the pellet was resuspended in 1 mL of extraction buffer 50 mM Tris-Cl, 1 mM EDTA, 4 mM $MgCl_2$, 5 mM DDT, 1 M NaCl, 10% glycerol, pH 7.5 with 2 μg/mL of leupeptin and 1 μg/mL of

pepstatin (added these inhibitors just prior to use). (50 mM Tris-Cl, 1 mM EDTA, 4 mM $MgCl_2$, 5 mM DDT, 1 M NaCl, 10% glycerol, pH 7.5). The cells were lysed by vortexing with glass beads (0.45-0.50 mm) at 4°C in presence of 2 µL of 1 M PMSF: 10 repeats of 20 seconds vortexing followed by 1 minute cooling on ice after each time. Then 2 µL of 1 M PMSF was added to the extract and the supernatant was discarded from glass beads by centrifugation for 10 minutes at 4°C. The crude extract was mixed with an equal volume of 50 mM Tris-Cl, 4 mM $MgCl_2$, 1 mM DTT, 10% glycerol, pH 7.5. Pre-equilibrated glutathione resin 100 µL was mixed with 2 mL of final extract (including added buffer in the last step). The resin and extract were mixed gently by rocking at 4°C for 3 hours. The unbound extract was removed, the resin was washed with 2 x 1 mL of 50 mM Tris-Cl, 4 mM $MgCl_2$, 1 mM DTT, 500 mM NaCl, 10% glycerol, pH 7.5 (wash buffer). The adsorbed protein was eluted from the resin by adding 2 mL of of freshly prepared elution buffer (wash buffer (1.98 mL) plus 2 M NaOH (20 µL) and solid glutathione (0.015 g)). The eluant was dialyzed against 20 mM Tris-Cl, 2 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT, 55 mM NaCl, 50% glycerol, pH 7.5 overnight, prior to storage at -20°C.

Regeneration System for Glutathione Resin

The glutathione agarose resin was regenerated by washing with 20 column volumes of phosphate-buffered saline supplemented with 3 M NaCl (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 3.14 M NaCl, pH 7.4) followed by 10 column volumes of wash buffer.

Protein Determination

Protein Concentration

A Bradford assay of the concentrated samples allowed determination of the total protein present in the dialyzed solution.[171] BSA was used as a protein standard.

SDS Gel Electrophoresis

SDS gel electrophoresis was used to monitor the expressed and purified products and it was carried out essentially as described by Maniatis.[168] Gels were stained with Coomassie Brilliant Blue R-250 for at least 30 minutes at room temperature and destained overnight.

Enzyme Assays

Reaction mixtures contained $NADP^+$ (0.20 μmoles, 0.15 mg), glucose-6-phosphate (14 μmoles, 4.3 mg), glucose-6-phosphate dehydrogenase (5 μg), ketone substrate (5 mM) and purified GST-fusion protein (10 – 100 μL, containing 5 – 50 μg) in 1.0 mL of 100 mM KP_i, pH 7.0. Reaction mixtures were incubated at 30°C. The extent of conversions and the values of enantio- and diastereomeric excess were determined by gas chromatography.

Organic Synthesis

Chemicals and General Procedures

Ketones **19**, **20a-e, 23a-c**, **28c,** and **35a** were obtained from commercial suppliers and used as received. α-Keto esters **28a** and **28b** were prepared by Fischer esterification of the commercially-available acids. β-Keto ester **20d,**[134] α-chloro β-keto esters **35b-d**[149] and α-fluoro β-keto esters **44a-d** [172] were prepared according to published procedures. In general, reaction products were purified by flash chromatography using 60Å silica gel. Thin layer chromatography was performed on plates of silica gel 60 from Aldrich.

Product characterization was done by GC-MS, ^{1}H and ^{13}C NMR, IR and by optical rotation. NMR spectra were obtained from instruments operating at 300 or 500 MHz for ^{1}H and 75 MHz for ^{13}C, respectively, and peaks were referenced to residual protonated solvent. IR spectra were recorded from thin films. Mass spectra (EI, 70 eV) were obtained from a bench-top GC/MS system equipped with a 0.32 mm × 30 m DB-17 column. Optical rotations were measured from $CHCl_3$ solutions using a Perkin-Elmer 241 or 341 polarimeter operating at room temperature. Analytical GC analyses were carried out with a 0.32 mm × 30 m DB-17 column for non-chiral separations and a 0.25 mm × 25 m Chirasil-Dex CB or a 0.25 × 25 m Chirasil-L-Val column for enantiomer separations. Samples for GC analysis of biotransformation reactions were prepared by mixing 200 μL of the reaction mixture with an equal volume of Et_2O. After vortex mixing, the organic layer was removed for analysis. Racemic alcohols were prepared from ketones by reduction with $NaBH_4$ and conditions that gave baseline resolution of all products were used for analyzing products from enzymatic reductions. Alcohols derived from β-keto ester **23b** required acetylation prior to GC analysis for resolution of the optical isomers.

Synthesis of Racemic β-Hydroxy Esters Standards

Racemic β-hydroxy esters used as standard in GC analysis were obtained by $NaBH_4$ reduction of the corresponding β-keto ester. Sodium borohydride (1 eqivalent) was dissolved in ethanol, then the β-keto ester was added to the stirred solution of $NaBH_4$ at room temperature. The progress of the reaction was followed by TLC (thin layer chromatography) until the reaction achieved completion. Excess sodium borohydride was quenched by adding saturated NH_4Cl, and then the reaction mixture was extracted with

diethyl ether (3 times). The organic layers were combined and dried with magnesium sulfate, and the solvent was removed by rotary evaporation.

Acetylation of β-Hydroxy Esters

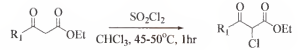
For most β-hydroxy esters, the different alcohol stereoisomers were resolved by chiral GC with no need for dervivatization. However, ethyl 2-ethyl-3-hydroxybutanoate required prior acetylation for reasonable resolution of the mixture of isomers. Quantitative acetylation was achieved with acetic anhydride in DMF, using DMAP (dimethyl amino pyridine) as a catalyst.[173] 5 mg of the β-hydroxy ester was dissolved in 500μL DMF, then 9μL of acetic anhydride and a small crystal of DMAP were added to the solution and the reaction was stirred overnight at room temperature. After this time, 1 mL of 0.5M $NaHCO_3$ was added and the reaction mixture was extracted with Et_2O (2 × 1 mL), then the combined organic layers were concentrated and analyzed by GC.

Synthesis of Substrates

Preparation of α-Chloro-β-Keto Esters 35b-d

Sulfuryl chloride (2g, 15 mmol) was added to a stirred solution of β-keto ester (**34b-d**) (15 mmol) in $CHCl_3$ (30 mL) at 45-50 °C during a period of 1 hour. The reaction mixture was diluted with ice-water (100 mL). The organic layer was separated, and the aqueous portion was extracted with CH_2Cl_2 (2 x100 mL). The resulting solution was dried by $MgSO_4$ and concentrated under reduced pressure to afford the α-chloro-β-keto esters. No further purification was necessary.

A mixture of α-chloro β-keto ester (**35a-d**) (6 mmol), NaH (60%, 0.48g, 12 mmol) was stirred for 3 hours in freshly distilled CH_2Cl_2 (60mL), then the fluorinating agent; (N-fluorobenzenesulfonimide) (2.8 g, 9 mmol) was added at room temperature.



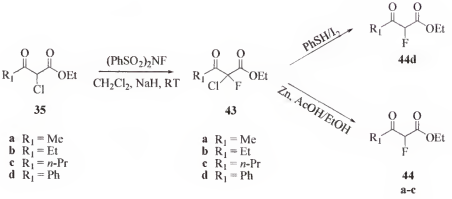
34 **35**

b R_1 = Et
c R_1 = *n*-Pr
d R_1 = Ph

	Isolated yields	
35b	2.27g	94%
35c	3.21g	89%
35d	2.52g	95%

Figure 7-1. Preparation of α-Chloro-β-Keto Esters **35b-d.**

Preparation of α-Fluoro-β-Keto Esters 44a-d



35 **43** **44d** **44 a-c**

a R_1 = Me
b R_1 = Et
c R_1 = *n*-Pr
d R_1 = Ph

	Isolated yields	
43a	0.30g	27%
43b	0.42g	36%
43c	0.56g	45%
43d	0.76g	52%

	Isolated yields	
44a	0.22g	75%
44b	0.10g	56%
44c	0.22g	72%
44d	0.13g	20%

Figure 7-2. Preparation of α-Fluoro-β-Keto Esters **44a-d**.

The reaction mixture was stirred overnight. After this, 200 mL of H_2O was added and resulting mixture was extracted with Et_2O (2 x 200 mL). The combined organic layers were dried over magnesium sulfate ($MgSO_4$), and concentrated under reduced pressure. The crude products were purified by flash chromatography (eluent TBME/hexane, 1/20) to afford pure α-chloro-α-fluoro β-ketoesters (**43a-d**).

Dechlorination of α-Chloro-α-Fluoro β-Keto Esters (43a-c)

Zinc powder (13.6 equivalents) was added to a solution of α-chloro-α-fluoro β-keto esters (**43a-c**) equivalent in 10% acetic acid in ethanol at room temperature. The reaction mixture was stirred under argon for 1 hour and then passed through a Celite-silica gel pad to remove unreacted zinc. The solid was washed with CH_2Cl_2 and H_2O. The aqueous layer was separated and extracted with several portions of CH_2Cl_2. The combined organic layers were washed with saturated aqueous $NaHCO_3$ and brine solution, dried with $MgSO_4$, and concentrated under reduced pressure. The crude products were purified by flash chromatography (eluent TBME/hexane, 1/20) to afford pure α-fluoro β-ketoesters (**44a-c**).

Dechlorination of Ethyl 2-Chloro-2-Fluoro-3-Oxo-Phenyl Propionate (43d)

A crystal of iodine (5-10mg) was added to a stirred solution of ethyl 2-chloro -2-fluoro-3-oxo-phenyl propionoate (**43d**, 0.75g, 3 mmol) and thiophenol (0.5mL) in dichloromethane (CH_2Cl_2, 5mL). The reaction mixture was stirred for 24 hours at room temperature and then the solvent was removed and the residue purified by flash chromatography (hexane was used to remove thiophenol and (phenyldithio)benzene, then the product was eluted by TBME/hexane, 1/20) to pure **44d**.

Preparation of Ethyl 5-Methyl-3-Oxohexanoate (20d)

To a three-neck round-bottom flask was added, under argon, diisopropylamine (19.3 mL, 13.7 mmol) in dry THF (117 mL). The resulting solution was cooled to 0°C, and then a solution of *n*-BuLi in hexane (2.5 M, 50 mL, 13.7 mmol) was added dropwise and the mixture was stirred for 10 min. This mixture was cooled to -70°C and dry ethyl acetate (6.7 mL, 6.84 mmol) was added and the mixture was stirred for 30 min. 3-methylbutanoyl chloride (8.36 mL, 6.84 mmol) was added dropwise in dry THF (6.8 mL). The mixture was stirred for 40 min, then an aqueous solution of ammonium chloride (5 mL) was added at -70°C, and the reaction was extracted with ether (3×50 mL). The organic layer was dried with $MgSO_4$. After evaporation of the solvent, the residue was purified using flash column chromatography (hexane: ethyl acetate (7:3)) to yield **20d** in 90%.

General Biotransformation with Commercial Baker's Yeast

Commercial baker's yeast (Fleishmann's) (9g/L) was incubated in an aqueous mixture solution containing 20g/L sucrose and β-hydroxypropyl cyclodextrin (1 equivalent) if required. The solution was stirred at 30°C for one hour. The substrate (5mM) was then added to the pre-incubated biotransformation as mixture with ethanol. The reaction mixture was stirred for 24 hours at 30°C. The resulting solution was filtered through Celite, and then the aqueous layer was extracted with ethyl acetate (3×50 mL). The combined organic layers were dried with $MgSO_4$, and concentrated under reduced pressure. The crude products were purified by flash chromatography.

NMR Data

α-Chloro-β-Keto Esters 35a-d

Ethyl 2-chloro 3-oxobutyrate (**35a**)

^{1}H NMR ($CDCl_3$,300 MHz) δ 1.31 (3H, t, *J*=7.17 Hz, CH_3CH_2); δ 2.38 (3H, s, $COCH_3$); δ 4.28 (2H, q, *J*=7.17 Hz CH_2CH_3) δ 4.76 (1H, s, COCHClCOO).

Ethyl 2-chloro 3-oxo-pentanoate (**35b**)

^{1}H NMR ($CDCl_3$,300 MHz) δ 1.12(3H, t, *J*=7.18 Hz, CH_3CH_2CO); δ 1.31 (3H, t, *J*=7.17 Hz, CH_3CH_2O); δ 2.75 (2H, dq, *J*=7.18 Hz $COCH_2CH_3$); δ 4.30 (2H, q, *J*=7.18 Hz OCH_2CH_3); δ 4.80 (1H, s, COCHClCOO).

Ethyl 2-chloro 3-oxo-hexanoate (**35c**)

^{1}H NMR ($CDCl_3$,300 MHz) δ 0.94(3H, t, *J*=7.18 Hz, CH_3CH_2O); δ 1.32 (3H, t, *J*=7.17 Hz, $CH_3CH_2CH_2O$); δ 1.66 (2H, q, *J*=7.18 Hz $CH_3CH_2CH_2O$); δ 2.69 (2H, m, $CH_3CH_2CH_2O$); δ 4.24 (2H, q, *J*=7.18 Hz OCH_2CH_3); δ 4.78 (1H, s, COCHClCOO).

Ethyl 2-chloro 3-oxo-phenylpropionoate (**35d**)

^{1}H NMR ($CDCl_3$,300 MHz) δ 1.25 (3H, t, *J*=7.17 Hz, CH_3CH_2); δ 4.28 (2H, q, *J*=7.17 Hz, CH_2 CH_3); δ 5.60 (1H, s, COCHClCOO); δ 7.50-8.00 (5H, m, C_6H_5).

α-Chloro-α-Fluoro-β-Keto esters 43a-d

Ethyl 2-chloro-2-fluoro 3-oxo-butyrate (**43a**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.37 (3H, t, *J*=7.17 Hz, CH_3CH_2); δ 2.45 (3H, d, J_{HF}=2.63 Hz $COCH_3$); δ 4.40 (2H, q, *J*=7.17 Hz CH_2CH_3).

Ethyl 2-chloro-2-fluoro 3-oxo-pentanoate (**43b**)

^{1}H NMR ($CDCl_3$,300 MHz) δ 1.17(3H, t, *J*=7.18 Hz, CH_3CH_2CO); δ 1.36 (3H, t, *J*=7.17 Hz, CH_3CH_2O); δ 2.82 (2H, m, $COCH_2CH_3$); δ 4.40 (2H, q, *J*=7.18 Hz OCH_2CH_3)

Ethyl 2-chloro-2-fluoro 3-oxo-hexanoate (**43c**).

^{1}H NMR ($CDCl_3$,300 MHz) δ 0.97(3H, t, *J*=7.18 Hz, CH_3CH_2O); δ 1.36 (3H, t, *J*=7.17 Hz, $CH_3CH_2CH_2O$); δ 1.70 (2H, q, *J*=7.18 Hz $CH_3CH_2CH_2O$); δ 2.76 (2H, m, $CH_3CH_2CH_2O$); δ 4.38 (2H, q, *J*=7.18 Hz OCH_2CH_3).

Ethyl 2-chloro-2-fluoro-3-oxo-phenylpropionoate (**43d**)

^{1}H NMR ($CDCl_3$,300 MHz) δ 1.27 (3H, t, *J*=7.17 Hz, CH_3CH_2); δ 4.36 (2H, q, *J*=7.17 Hz, CH_2CH_3); δ 7.50-8.10 (5H, m, C_6H_5).

α-Fluoro-β-Keto esters 44a-d

Ethyl 2-fluoro 3-oxo-butyrate (**44a**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.33 (3H, t, *J*=7.17 Hz, CH_3CH_2); δ 2.36 (3H, d, J_{HF}=4.10 Hz $COCH_3$); δ 4.32 (2H, q, *J*=7.17 Hz CH_2CH_3) δ 5.20 (1H, d, J_{HF}=49.47 Hz COCHFCOO).

Ethyl 2-fluoro 3-oxo-pentanoate (**44b**)

^{1}H NMR ($CDCl_3$,300 MHz) δ 1.14 (3H, t, *J*=7.18 Hz, CH_3CH_2CO); δ 1.33 (3H, t, *J*=7.17 Hz, CH_3CH_2O); δ 2.70 (2H, m, CH_3 CH_2CO); δ 4.30 (2H, q, *J*=7.18 Hz OCH_2CH_3); δ 5.20 (1H, d, J_{HF}=48.49 Hz COCHFCOO).

Ethyl 2-fluoro 3-oxo-hexanoate (**44c**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.94(3H, t, *J*=7.18 Hz, CH_3CH_2O); δ 1.33 (3H, t, *J*=7.17 Hz, $CH_3CH_2CH_2O$); δ 1.66 (2H, q, *J*=7.18 Hz $CH_3CH_2CH_2O$); δ 2.66 (2H, m, $CH_3CH_2CH_2O$); δ 4.30 (2H, q, *J*=7.18 Hz OCH_2CH_3) δ 5.20 (1H, d, J_{HF}=49.66 Hz COCHFCOO).

Ethyl 2-fluoro 3-oxo-phenyl-propionoate (**44d**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.26 (3H, t, J=7.17 Hz, CH_3CH_2); δ 4.29 (2H, q, J=7.17 Hz, CH_2CH_3); δ 5.85 (1H, d, J_{HF}=48.74 Hz COCHFCOO); δ 7.40-8.20 (5H, m, C_6H_5).

α-Chloro β-Hydroxy Esters

Ethyl 2-chloro-3-hydroxy pentanoate (**36-39a**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.00-1.40 (6H, m, CH_3CH_2O, $CH3CH_2CHOH$); δ 1.60 (2H, m, CH_3CH_2CHOH); δ 3.70 (1H, m, CH_3CH_2CHOH); δ 4.20 (2H, q, J=7.18 Hz OCH_2CH_3) δ 4.30 (1H, m, CHCl).

Ethyl 2-chloro-3-hydroxy hexanoate (**36-39b**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.96(3H, t, J=7.18 Hz, CH_3CH_2O); δ 1.33 (3H, t, J=7.17 Hz, $CH_3CH_2CH_2O$); δ 1.40-1.70 (4H, m, $CH_3CH_2CH_2O$); δ 3.70 (1H, m, CHOH); δ 4.20 (2H, q, J=7.18 Hz OCH_2CH_3) δ 4.30 (1H, m, CHCl).

Ethyl 2-chloro-3-hydroxy-phenylpropionoate (**36-39c**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.12 (3H, t, CH_3CH_2O, J=7.18 Hz); δ 4.08 (2H, q, J=7.18 Hz CH_3CH_2O); δ 4.48 (1H, d, CH_3CH_2CHOH) δ 4.76 (1H, m, CHCl); δ 7.30-7.50 (5H, m, Ph).

Ethyl 2-choro-3-hydroxy butyrate (**36-39d**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.17 (3H, t, J=7.18 Hz, CH_3CH_2O); δ 1.30 (3H, d, CH_3CHOH); δ 3.95 (1H, m, CH_3CHOH) δ 4.15 (2H, q, CH_3CH_2O) δ 4.35 (1H, m, CHCl).

α-Fluoro β-Hydroxy Esters

Ethyl 2-fluoro-3-hydroxy butyrate (**45-48a**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.20-1.40 (6H, m, CH_3CH_2O, CH_3CHOH); δ 3.72 (1H, m, CH_3CHOH); δ 4.30 (2H, q, *J*=7.17 Hz CH_2CH_3O) δ 4.80 (1H, m, CHF).

Ethyl 2-fluoro-3-hydroxy pentanoate (**45-48b**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.20-1.40 (6H, m, CH_3CH_2O, CH_3CH_2CHOH); δ 1.66 (2H, m, CH_3CH_2CHOH); δ 3.70 (1H, m, CH_3CH_2CHOH); δ 4.30 (2H, q, *J*=7.18 Hz OCH_2CH_3) δ 4.86 (1H, m, CHF).

Ethyl 2-fluoro-3-hydroxy hexanoate (**45-48c**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.96(3H, t, *J*=7.18 Hz, CH_3CH_2O); δ 1.33 (3H, t, *J*=7.17 Hz, $CH_3CH_2CH_2O$); δ 1.40-1.70 (4H, m, $CH_3CH_2CH_2O$); δ 4.00(1H, m, CHOH); δ 4.30 (2H, q, *J*=7.18 Hz OCH_2CH_3) δ 4.85 (1H, m, CHF).

Ethyl 2-fluor-3-hydroxy phenyl-propionoate (**45-48d**)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.17 (3H, t, CH_3CH_2O, *J*=7.18 Hz); δ 4.15 (2H, q, *J*=7.18 Hz CH_3CH_2O); δ 4.24 (1H, m, CH3CH_2CHOH) δ 4.73 (1H, dd, CHF); δ 7.30-7.50 (5H, m, Ph).

Ethyl 5-Methyl-3-Oxohexanoate (20d)

^{1}H NMR ($CDCl_3$, 300MHz) δ 0.93 (d, J=6.7 Hz, 6H), 1.28 (t, J=7.1 Hz, 3H), 2.19 (non, J=6.7 Hz, 1H), 2.41 (d, J=6.7 Hz, 2H), 3.4 (s, 2H), 4.23 (q, J=7.1 Hz, 2H).

Ethyl 3-hydroxy-5-methyl-hexanoate

^{1}H NMR ($CDCl_3$, 300MHz) δ 0.92 (d, J=6.6 Hz, 6H); 1.18 (ddd, J=4.5; 8.6; 13.8 Hz, 1H); 1.27 (t, J=7 Hz, 3H); 1.49 (ddd, J=5.5; 8.8; 13.8 Hz, 1H); 1.63–1.90 (m, 1H), 2.37 (dd, J=8.4; 16.5 Hz, 1H); 2.50 (dd, J=3.6; 16.5 Hz, 1H), 2.9 (brs, 1H, OH), 4.17 (q, J=7 Hz, 2H); 4.06 (quint., J=4.2 Hz, 1H).

APPENDIX A
BIOCATALYTIC REDUCTIONS OF REPRESENTATIVE SUBSTRATES

Table A-1. Biocatalytic reductions of representative β-keto esters.

Yeast gene	Plasmid	 (*R*) : (*S*)	(*R*) : (*S*)	(*R*) : (*S*)	(*R*) : (*S*)	(*R*) : (*S*)
YJR096w	pIK9	<1 : >99	---[a]	---	---	<1 : >99
YDL124w	pIK8	<1 : >99	---	---	---	<1 : >99
YBR149w	pIK12	<1 : >99	---	---	---	60 : 40
YOR120w	pIK30	<1 : >99	<1 : >99	<1 : >99	---	>99 : <1
YHR104w	pIK29	---	---	---	---	<1 : >99
YDR368w	pIK4	<1 : >99	<1 : >99	<1 : >99	---	>99 : <1
YGL185c	pAKS1	---	---	---	---	<1 : >99
YNL274c	pIK13	---	---	---	---	<1 : >99
YPL275w	pIK18	<1 : >99	---	---	---	>99 : <1
YPL113c	pIK15	<1 : >99	<1 : >99	16 : 84	---	>99 : <1
YLR070c	pIK23	<1 : >99	---	---	--	41 : 59
YAL060w	pTM3	<1 : >99	<1 : >99	<1 : >99	>99 : <1	>99 : <1
YAL061w	pIK28	---	---	---	---	<1 : >99
YGL157w	pIK7	<1 : >99	<1 : >99	<1 : >99	---	>99 : <1
YDR541c	pIK5	<1 : >99	<1 : >99	<1 : >99	>99 : <1	>99 : <1
YGL039w	pIK6	<1 : >99	<1 : >99	<1 : >99	>99 : <1	>99 : <1
YNL331c	pIK11	13 : 87	---	23 : 77	---	58 : 42

Table A-1 Contnued.

YCR107w	pIK10	<1 : >99	38 : 62	---	---	71 : 29
YOL151w	pIK3	<1 : >99	<1 : >99	<1 : >99	---	>99 : <1
Yeast Cells	---	3 : 97	54 : 46	>99 : <1	10 : 90	23 : 77

[a] < 20% conversion after 24 hours

Table A-2. Biocatalytic reductions of representative α-keto esters.

Yeast gene	**Plasmid**	 (*R*) : (*S*)	 (*R*) : (*S*)	 (*R*) : (*S*)
YJR096w	pIK9	64 : 36	>99 : <1	73 : 27
YDL124w	pIK8	>99 : <1	>99 : <1	>99 : <1
YBR149w	pIK12	5 : 95	18 : 82	21 : 79
YOR120w	pIK30	<1 : >99	---[a]	>99 : <1
YHR104w	pIK29	---	---	---
YDR368w	pIK4	<1 : >99	93 : 7	>99 : <1
YGL185c	pAKS1	>99 : <1	>99 : <1	>99 : <1
YNL274c	pIK13	50 : 50	60 : 40	60 : 40
YPL275w	pIK18	<1 : >99	---	---
YPL113c	pIK15	<1 : >99	>99 : <1	20 : 80
YLR070c	pIK23	---	--	45 : 55
YAL060w	pTM3	>99 : <1	>99 : <1	>99 : <1
YAL061w	pIK28	---	---	---
YGL157w	pIK7	>99 : <1	>99 : <1	>99 : <1
YDR541c	pIK5	>99 : <1	>99 : <1	>99 : <1
YGL039w	pIK6	>99 : <1	>99 : <1	>99 : <1

Table A-2 Continued.

YNL331c	pIK11	26 : 74	50 : 50	50 : 50
YCR107w	pIK10	5 : 95	23 : 77	27 : 73
YOL151w	pIK3	24 : 76	>99 : <1	<1 : >99
Yeast Cells	---	11 : 89	12 : 88	81 : 19

[a] < 20% conversion after 24 hours

Table A-3. Biocatalytic reductions of representative α-substituted β-keto esters.

Yeast gene	**Plasmid**	 *anti*-**24a** : *syn*-**25a** *anti*-**26a** : *syn*-**27a**	 *anti*-**24b** : *syn*-**25b** *anti*-**26b** : *syn*-**27b**	 *anti*-**24c** : *syn*-**25c** *anti*-**26c** : *syn*-**27c**
YJR096w	pIK9	---	---	---
YDL124w	pIK8	<1 : >99 : <1 : <1	<1 : >99 : <1 : <1	---
YBR149w	pIK12	29 : 71 : <1 : <1	6 : 94 : <1 : <1	27 : 73 : <1 : <1
YOR120w	pIK30	<1 : >99 : <1 : <1	<1 : >99 : <1 : <1	<1 : >99 : <1 : <1
YHR104w	pIK29	>99 : <1 : <1 : <1	---	---
YDR368w	pIK4	<1 : >99 : <1 : <1	<1 : >99 : <1 : <1	<1 : >99 : <1 : <1
YGL185c	pAKS1	47 : 53 : <1 : <1	---	---
YNL274c	pIK13	48 : 52 : <1 : <1	---	---
YPL275w	pIK18	19 : 81 : <1 : <1	---	32 : 68 : <1 : <1
YPL113c	pIK15	20 : 80 : <1 : <1	36 : 64 : <1 : <1	75 : 25 : <1 : <1
YLR070c	pIK23	43 : 57 : <1 : <1	--	---
YAL060w	pTM3	52 : 48 : <1 : <1	82 : 13 : 5 : <1	95 : 5 : <1 : <1
YAL061w	pIK28	59 : 41 : <1 : <1	---	---
YGL157w	pIK7	86 : 14 : <1 : <1	85 : 15 : <1 : <1	74 : 26 : <1 : <1

Table A-3 Continued.

YDR541c	pIK5	52 : 48 : <1 : <1	86 : 14 : <1 : <1	97 : 3 : <1 : <1
YGL039w	pIK6	30 : 70 : <1 : <1	47 : 53 : <1 : <1	83 : 17 : <1 : <1
YNL331c	pIK11	35 : 65 : <1 : <1	36 : 64 : <1 : <1	---
YCR107w	pIK10	14 : 86 : <1 : <1	---	32 : 68 : <1 : <1
YOL151w	pIK3	6 : 94 : <1 : <1	42 : 58 : <1 : <1	87 : 13 : <1 : <1
Yeast Cells	---	<1 : >99 : <1 : <1	45 : 55 : <1 : <1	80 : 20 : <1 : <1

[a] < 20% conversion after 24 hours

Table A-4. Biocatalytic reductions of representative α-chloro-β-keto esters.

Yeast gene	**Plasmid**	 *anti*-**36a**: *syn*-**37a** *anti*-**38a**: *syn*-**39a**	 *anti*-**36b**: *syn*-**37b** *anti*-**38b**: *syn*-**39b**	 *anti*-**36c**: *syn*-**37c** *anti*-**38c**: *syn*-**39c**	 *anti*-**36d**: *syn*-**37d** *anti*-**38d**: *syn*-**39d**
YJR096w	pIK9	70:30:<1:<1	<1:22:<1:78	---[a]	<1:<1:<1:>99
YDL124w	pIK8	8:92:<1:<1	<1:12:33:55	---	<1:<1:<1:>99
YBR149w	pIK12	5:95:<1:<1	<1:>99:<1:<1	<1:>99:<1:<1	---
YOR120w	pIK30	<1:>99:<1:<1	<1:>99:<1:<1	---	---
YHR104w	pIK29	92:8:<1:<1	---	---	---
YDR368w	pIK4	7:93:<1:<1	<1:>99:<1:<1	6:94:<1:<1	---
YGL185c	pAKS1	56:44:<1:<1	<1:40:<1:60	---	<1:<1:<1:>99
YNL274c	pIK13	27:73:<1:<1	<1:40:<1:60	---	<1:<1:<1:>99
YPL275w	pIK18	43:47:<1:<1	---	---	---
YPL113c	pIK15	18:82:<1:<1	25:75:<1:<1	---	---
YLR070c	pIK23	43:57:<1:<1	---	---	---

Table A-4 Continued.

YAL060w	pTM3	74:26:<1:<1	95:5:<1:<1	>99:<1:<1:<1	--
YAL061w	pIK28	81:19:<1:<1	<1:<1:<1:>99	---	---
YGL157w	pIK7	97:3:<1:<1	>99:<1:<1:<1	>99:<1:<1:<1	---
YDR541c	pIK5	82:18:<1:<1	87:13:<1:<1	>99:<1:<1:<1	---
YGL039w	pIK6	16:84:<1:<1	90:10:<1:<1	97:3:<1:<1	10:90:<1:<1
YNL331c	pIK11	33:67:<1:<1	<1:>99:<1:<1	<1:>99:<1:<1	11:89:<1:<1
YCR107w	pIK10	6:94:<1:<1	<1:>99:<1:<1	<1:>99:<1:<1	---
YOL151w	pIK3	16:84:<1:<1	---	---	---
Yeast Cells	---	50:50:<1:<1	46:6:42:6	70:10:18:2	20:11:45:24

[a] < 20% conversion after 24 hours

Table A-5. Biocatalytic reductions of representative α-fluoro-β-keto esters (only relative configuration was available).

Yeast gene	**Plasmid**	 *anti-* **45a,47a** *syn-* **46a,48a**	 *anti-* **45b,47b** *syn-* **46b,48b**	 *anti-* **45c,47c** *syn-* **46c,48c**	 *anti-* **45d,47d** *syn-* **46d,48d**
YJR096w	pIK9	29 : 71	7 : 93	3 : 97	10 : 90
YDL124w	pIK8	7 : 93	<1 : >99	<1 : >99	<1 : >99
YBR149w	pIK12	38 : 62	40 : 60	35 : 65	80 : 20
YOR120w	pIK30	93 : 7	50 : 50	60 : 40	68 : 32
YHR104w	pIK29	64 : 36	40 : 60	---[a]	---
YDR368w	pIK4	57 : 43	36 : 64	17 : 83	28 : 72
YGL185c	pAKS1	37 : 63	2 : 98	<1 : >99	<1 : >99
YNL274c	pIK13	33 : 67	8 : 92	4 : 96	<1 : >99

Table A-5 Continued.

YPL275w	pIK18	47 : 53	33 : 67	15 : 85	---
YPL113c	pIK15	15 : 85	17 : 83	10 : 90	17 : 83
YLR070c	pIK23	52 : 48	15 : 85	10 : 90	<1 : >99
YAL060w	pTM3	4 : 96	<1 : >99	<1 : >99	<1 : >99
YAL061w	pIK28	50 : 50	11 : 89	<1 : >99	<1 : >99
YGL157w	pIK7	3 : 97	5 : 95	<1 : >99	2 : 98
YDR541c	pIK5	4 : 96	<1 : >99	<1 : >99	40 : 60
YGL039w	pIK6	18 :82	2 : 98	<1 : >99	37 : 63
YNL331c	pIK11	48 : 52	45 : 55	55 : 45	70 : 30
YCR107w	pIK10	32 : 68	42 : 58	45 : 55	---
YOL151w	pIK3	20 : 80	15 : 85	6 : 94	25 : 75
Yeast Cells	---	22 : 78	10 : 90	26 : 74	5 : 95

[a] < 20% conversion after 24 hours

Table A-6. Concentrations of purified GST-yeast reductases expressed in *E. coli.*

GST-Yeast ORF	**Concentration [mg/mL]**
YJR096w	3
YDL124w	2
YBR149w	0.4
YOR120w	1.5
YHR104w	1.5
YDR368w	2
YGL185c	0.5
YNL274c	2
YPL275w	0.8

Table A-6. Continued.

YPL113c	0.5
YLR070c	0.4
YAL060w	0.5
YAL061w	0.4
YGL157w	2
YDR541c	0.8
YGL039w	2.8
YNL331c	0.8
YCR107w	0.4
YOL151w	3

REFERENCES

1. Faber, K. *Biotransformations in Organic Chemistry*; 4th ed.; Springer: Berlin, 2000.

2. Noyori, R. *Asymmetric Catalysis in Organic Synthesis*; 1st ed.; John Wiley & Sons, Inc.: New York, 1994.

3. Zhou, B.; Gopalan, A. S.; VanMiddlesworth, F.; Shieh, W. –R.; Sih, C. J. *J. Am. Chem. Soc*. 1983, *105*, 5925-5926.

4. Kitamura, M.; Ohkuma, T.; Takaya, H.; Noyori, R. *Tetrahedron Lett*. 1988, *29*, 1555-1556.

5. Karanewsky, D. S.; Badia, M. C.; Ciosek, C. P.; Robl, J. F.; Sofia, M. J.; Simpkins, L. M.; DeLange, B.; Harrity, T. W.; Biller, S. A.; Gorden, E. M. *J. Med. Chem*. 1990, *33*, 2925-2956.

6. Keinan, E.; Sinha, S. C.; Sinha- Bagchi, A. *J. Org. Chem*. 1992, *57*, 3631-3636.

7. Cainelli, G.; Galleti, P.; Giacomini, D. *Tetrahedron Lett*. 1998, *39*, 7779-7782.

8. Shafiee, A.; Motamedi, H.; King, A. *Appl. Microbiol. Biotechnol*. 1998, *49*, 709-717.

9. Blaser, H.-U.; Jalett, H.-P.; Spindler, F. *J. Mol. Catalysis A: Chemical* 1996, *107*, 85-94.

10. Kaluzna, I. A.; Andrew, A. A.; Bonilla, M.; Martzen, M. R.; Stewart, J. D. *J. Mol. Cat. B Enzymatic* 2002, *17*, 101-105.

11. Itsuno, S. *Organic Reactions* 1998, *52*, 395-576.

12. Brown, H. C.; Cho, B. T.; Park, W. S. *J. Org. Chem.* 1988, *53*, 1231-1238.

13. Naota, T.; Takaya, H.; Murahashi, S.-I. *Chem. Rev*. 1998, *98*, 2599-2660.

14. Genet, J. P.; Ratovelomanana-Vidal, V.; Cano de Andrade, M. C.; Pfister, X.; Guerreiro, P.; Lenoir, J. Y. *Tetrahedron Lett.* 1995, *36*, 4801-4804.

15. Buse, C. T.; Heathcock, C. H. *J. Am. Chem. Soc.* 1977, *99*, 2337.

16. Frater, G.; Muller, U.; Gunther, W. *Tetrahedron* 1984, *40*, 1269.

17. Genet, J. P. *Reduction in Organic Synthesis. Recent Advances and Practical Applications;* Abdel-Magid, A. F. , Ed.; American Chemical Society: Washington, DC, 1996, Vol. 641, p. 31-51.

18. Soai, K.; Yamanoi, T.; Hikima, H.; Oyamada, H. *J. Chem. Soc. Chem. Commun.* 1985, 138.

19. Marcantoni, E.; Alessandrini, S.; Malavolta,M. *J. Org. Chem.* 1999, *64*, 1986-1992.

20. Oishi, T.; Nakata, T. *Acc. Chem. Res.* 1984, *17*, 338-344.

21. Vasconcellos, M. L.; d'Angelo, J.; Desmaele, D.; Costa, P. R. R.; Potin, D. *Tetrahedron: Asymmetry* 1991, *2*, 353-356.

22. Burk, M. J. Gross, M. F.; Harper, T. G. P.; Kalberg, C. S.; Lee, J. R.; Martinez, J. P. *Pure & Appl. Chem.* 1996, *68*, 37-44.

23. Noyori, R.; Ohkuma, T.; Kitamura, M. *J. Am. Chem. Soc.* 1987, *109*, 5856-5858.

24. Burk, M. J.; Harper, T. G. P.; Kalberg, C. S. *J. Am. Chem. Soc.* 1995, *117*, 4423.

25. Ratovelomanana-Vidal, V.; Genet, J. P *J. Organometallic Chem.* 1998, *567*, 163-171.

26. Noyori, R.; Ikeda, T.; Ohkuma, T.; Widhalm, M.; Kitamura, M.; Takaya, H.; Akutagawa, S.; Sayo, N.; Saito, T.; Taketomi, T.; Kumobayashi, H. *J. Am. Chem. Soc.* 1989, *111*, 9134.

27. Ohkuma, T.; Kitamura, M.; Noyori, R. *Catalytic Asymmetric Synthesis;* 2nd ed.; Wiley-VCH, Inc.: New York, 2000.

28. Halle, R.; Breheret, A.; Schultz, E.; Pinel, C.; Lemaire, M. *Tetrahedron: Asymmetry* 1997, *8*, 2101-2108.

29. Heathcock, C. H. *Aldrichimica Acta* 1990, *23,* 99-111.

30. Van Draanen, N. A.; Arseniyadis, S.; Crimmins, N. T.; Heathcock, C. H. *J. Org. Chem.* 1991, *56*, 2499.

31. *Enzyme Nomenclature*; San Diego; Academic Press, 1992.

32. Chen, C.-S.; Zhou, B.-N.; Girdaukas, W.-R.; Shieh, W.-R.; VanMiddlesworth, F.; Gopalan, A. S.; Sih, C. J. *Bioorg. Chem.* 1984, *12,* 98-117.

33. Prelog, V. *Pure Appl. Chem.* 1968, *9*, 119-130.

34. Jones, J. B. *Tetrahedron* 1986, *42*, 3351-3403.

35. Davies, J.; Jones, J. B.; *J. Am. Chem. Soc.* 1979, *101*, 5405-5410.

36. Lam, L. K. P.; Gair, I. A.; Jones, J. B. *J. Org. Chem.* 1988, *53*, 1611-1615.

37. Jones, J. B.; Sih, C. J. Perlman, D. *Applications of Biochemical in Organic Chemistry*; Wiley: New York, 1976.

38. Bradshaw, C. W.; Fu, H.; Shen, G.-J.; Wong, C.-H. *J. Org. Chem.* 1992, *57*, 1526-1532.

39. Sih, C. J.; Chen C.-S. Angew. *Chem. Int. Ed. Engl.* 1984, *23*, 570.

40. Servi, S.; *Synthesis* 1990, *1*, 1-25.

41. Csuk, R.; Glanzer, B. *Chem. Rev.* 1991, *91*, 49-97.

42. Seebach, D.; Sutter, M. A.; Weber, R. H.; Zuger, M. F. *Org. Synth.* 1985, *63*, 1.

43. Mochizuki, N.; Sugai, T.; Ohta, H. *Biosci. Biotech. and Biochem.* 1994, *58*, 1666-11670.

44. Shieh, W.-R.; Gopalan, A. S.; Sih, C. J. *J. Am. Chem. Soc.* 1985, *107,* 2993-2994.

45. Barry, J.; Kagan, H. B. *Synthesis* 1981, 453-455.

46. Deol, B. S.; Ridley, D. D.; Simpson, G. W. *Aust. J. Chem.* 1976, *29*, 2459.

47. Seebach, D.; Roggo, S.; Maetzke, T.; Braunschweiger, H.; Cercus, J.; Krieger, M. *Helv. Chim. Acta* 1987, *70*, 1605.

48. Iriuchijima, S.; Ogawa, M. *Synthesis* 1982, 41.

49. Drochner, D.; Muller, M. *Eur. J. Org. Chem.* 2001, 211-215.

50. Wolberg, M.; Hummel, W.; Muller, M. *Chem. Eur. J.* 2001, *7*, 4562-4571.

51. Fujisawa, T.; Onogawa, Y.; Sato, A.; Mitsuya, T.; Shimizu, M. *Tetrahedron* 1998, *54,* 4267-4276.

52. Carvalho, M. D.; Okamoto, M. T.; Moran, P. J. S.; Rodrigues, J. A. R. *Tetrahedron* 1991, *47*, 2073-2080.

53. Zhou, B.-N.; Gopalan, A. S.; VanMiddlesworth, F.; Shieh, W.-R.; Sih, C. J. *J. Am. Chem. Soc.* 1983, *105*, 5925-5926.

54. Wipf, B.; Kupfer, E.; Bertazzi, R.; Leuenberger, H. G. W. *Helv. Chim. Acta* 1983, *66*, 485-488.

55. Kometani, T.; Yoshii, H.; Takeuchi, Y.; Matsuno, R. *J. Ferm. Bioeng.* 1993, *76*, 33-37.

56. Nakumara, K.; Kondo, S.; Kawai, Y.; Ohno, A. *Tetrahedron Lett.* 1991, 7075-7078.

57. Medson, C.; Smallridge, A. J.; Trewhella, M. A. *Tetrahedron: Asymmetry* 1997, *8*, 1049-1054.

58. Nakumara, K.; Kawai, Y.; Oka, S.; Ohno, A. *Tetrahedron Lett.* 1989, 2245-2246.

59. Guo, Z.-W.; Sih, C. J. *J. Am. Chem. Soc.* 1989, *111*, 6836-6841.

60. Kawai, Y.; Kondo, S.; Tsujimoto, M.; Nakumara, K.; Ohno, A. *Bull. Chem. Soc. Jpn.* 1994, *67*, 2244-2247.

61. Hayakawa, R.; Shimizu, M.; Fusjiawa, T. *Tetrahedron: Asymmetry* 1997, *8*, 3201-3204.

62. Dao, D. H.; Okamura, M.; Akasaka, T.; Kawai, Y.; Kouichi, H.; Ohno, A. *Tetrahedron: Asymmetry* 1998, *9*, 2725-2737.

63. Stewart, J. D.; Reed, K. W.; Martinez, C. A.; Zhu, J.; Chen, G.; Kayser, M. M. *J. Am. Chem. Soc.* 1998, *120*, 3541-3548.

64. Burdette, D.S.; Secundo, F.; Phillips, R.S.; Dong, J.; Scott, R.A.; Zeikus, J.G. *Biochem. J.* 1997, *326*, 717-724.

65. Rodriguez, S.; Kayser, M. M.; Stewart, J. D. *J. Am. Chem. Soc.* 2001, *123*, 1547-1555.

66. Ema, T.; Sugiyama, Y.; Fukumoto, M.; Moriya, H.; Cui, J.-N.; Sakai, T.; Utaka, M. J. *Org. Chem.* 1998, *63*, 4996-5000.

67. Cui, J.-N.; Teraoka, R.; Ema, T.; Sakai, T.; Utaka, M. *Tetrahedron Lett.* 1997, *38*, 3021.

68. Nakumara, K.; Yamanaka, R.; Matsuda, T.; Harada, T. *Tetrahedron Asymmetry* 2003, *14*, 2659-2681.

69. Nakamura, K.; Kawai, Y.; Miyai, T.; Honda, S.; Nakajima, N.; Ohno, A. *Bull. Chem. Soc. Jpn.* 1991, *64*, 1467-1470.

70. Nakamura, K.; Kawai, Y.; Nakajima, N.; Ohno, A. *J. Org. Chem.* 1991, *56*, 4778-4783.

71. Sybesma, W. F. H.; Straathof, A. J. J.; Jongejan, J. A.; Pronk, J. T.; Heijnen, J. J. *Biocatalysis and Biotransformation* 1998, *16,* 95-134.

72. Stewart, J. D.; Rodriguez, S.; Kayser, M. M. *Enzyme Technology for Pharmaceuticals and Biotechnological Applications*; Marcel Dekker, Inc.: New York, 2001.

73. Heidlas, E.; Engel, K.; Tressl, R. *J. Biochem.* 1988, 633-639.

74. Furuichi, A; Akita, H.; Matsukura, H.; Oishi, T.; Horikoshi, K. *Agric. Biol. Chem.* 1985, *49*, 2563-2570.

75. Shieh, W.-R.; Sih, C. J. *Tetrahedron: Asymmetry* 1993, *4*, 1259-1269.

76. Mewes, H. W.; Albermann, K.; Bahr, M.; Frishman, D.; Gleissner, A.; Hani, J.; Heumann, K.; Kleine, K.; Maierl, A.; Oliver, S. G.; Pfeiffer, F.; Zollner, A. *Nature* 1997, *387*, 7-8.

77. Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang J.; Zhang Z.; Miller, W.; Lipman D. J. *Nucleic Acids Res.* 1997, *25*, 3389-3402.

78. Stewart, J. D. *Curr. Opin. Biotechnol.* 2000, *11*, 363-368.

79. Albers, E.; Laizé, V.; Blomberg, A.;Hohmann, S.; Gustafsson, L. *J. Biol.Chem.* 2003, *278*, 10264-10272.

80. Overkamp, M. K.; Kötter, P.; van der Hoek , R.; Schoondermark-Stolk , S.; Luttik, M.A.H.; van Dijken , J.P.; Pronk, J. T. *Yeast* 2002, *19*, 509-520.

81. Delneri, D.; Gardner, D. C.; Bruschi, C. V.; Oliver, S. G. *Yeast* 1999, *15*, 1681-1689.

82. Delneri, D.; Gardner, D. C.; Bruschi, C. V.; Oliver, S. G. *Genetics* 1999, *153,* 1591-1600.

83. Kim, S. T.; Huh, W. K.; Lee, B. H.; Kango, S. *Biochim. Biophys. Acta* 1998, *1429*, 29-39.

84. Kuhn, A.; van Zyl, C.; van Tonder, A.; Prior, B. A. *Appl. Environ. Microbiol.* 1995, *61*, 1580-1585.

85. Hur, E.; Wilson, D. K. *Acta Crystallogr*. 2000*, D56*, 763-765.

86. Nakamura, K.; Kondo, S.; Kawai, Y.; Nakajima, N.; Ohno, A. *Biosci. Biotechnol. Biochem.* 1997, *61*, 375-377.

87. Rodriguez, S. *Doctoral Dissertation* 2000, University of Florida.

88. Rodriguez, S.; Schroeder, K. T.; Kayser, M. M.; Stewart, J. D. *J. Org. Chem.* 2000, *65*, 2586-2587.

89. Nakajima, N.; Ishihara, K.; Kondo, S.; Tsuboi, S.; Utaka, M.; Nakamura, K. *Biosci. Biotechnol. Biochem.* 1994, *58*, 2080-2081.

90. Katz, M.; Frejd, T.; Hahn-Hagerdal, B.; Gorwa-Grauslund, M. F. *Biotechnol. Bioeng.* 2003, *84*, 573-582.

91. Nakamura, K.; Kondo, S.; Kawai, Y.; Nakajima, N.; Ohno, A. *Biosci. Biotechnol. Biochem.* 1994, *58*, 2236-2240.

92. Traff, K. L.; Jonsson, L. J.; Hahn-Hagerdal, B. *Yeast* 2002, *19*, 1233-1241.

93. Harrison, D. H.; Bohren, K. M.; Ringe, D.; Petsko, G. A.; Gabbay, K. H. *Biochemistry* 1994, *33*, 2011-2020.

94. Ellis, E. M. *FEMS Microbiology Letters* 2002, *216*, 123-131.

95. Jeong, E. Y.; Kim, S. I.; Lee, H. *FEMS Microbiology Letters* 2002, *209*, 223-228.

96. Persson, B.; Zigler, J. S.; Jornvall, H. *Eur. J. Biochem.* 1994, *226*, 15-22.

97. Gonzalez, E.; Fernandez, M. R.; Larroy, C.; Pares, X.; Biosca, J. A. *Chemico-Biological Interactions* 2001, *130-132*, 425-434.

98. Larroy, C.; Fernandez, M. R.; Gonzalez, E.; Pares, E.; Biosca, J. A. *Biochem. J.* 2002, *361*, 163-172.

99. Larroy, C.; Fernandez, M. R.; Gonzalez, E.; Pares, X.; Biosca J. A. *Chemico-Biological Interactions* 2003, 143-144, 229-238.

100. Larroy, C.; Pares, X.; Biosca, J. A. *Eur. J. Biochem.* 2002, *269*, 5738-5745.

101. Sarthy, A.; Schopp, C.; Idler, K. B. *Gene* 1994, *140*, 121-126.

102. Richard, P.; Toirani, M. H.; Penttila, M. *FEBS Lett.* 1999, *457*, 135-138.

103. Filling, C.; Berndt, K. D.; Benach, J.; Knapp, S.; Prozorowski, T.; Nordling, E.; Ladenstein,R.; Jornvall, H.; Oppermann, U. *J. Biol. Chem.* 2002, *277*, 25677-25684.

104. Ishihara, K.; Nakajima, N.; Tsuboi, S.; Utaka, M. *Bull. Chem. Soc. Jpn.* 1994, *67*, 3314-3319.

105. Rodriguez, S.; Kayser, M. M.; Stewart, J. D. *Org. Lett.* 1999, *8*, 1153-1155.

106. Katz, M.; Hahn-Hagerdal, B.; Gorwa-Grauslund, M. F. *Enzyme and Microbial Technology* 2003, *33*, 163-172.

107. Goldberg, J. D.; Yoshida, T.; Brick, P. *J. Mol. Biol*. 1994, *236*, 1123-1140.

108. Kallwass, H. K. W. *Enzyme Microb. Technol*. 1992, *14*, 28-35.

109. Bernard, N.; Johnsen, K.; Ferain, T.; Garmyn, D.; Hols,P.; Holbrook, J. J.; Delcour, J. *Eur. J. Biochem*. 1994, *224*, 439-446.

110. Simon, E. S.; Plante, R.; Whitesides, G. M. *Appl. Biochem. Biotech*. 1989, *22*, 169.

111. Wong, C. H.; Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry*; 1st ed. ; Pergamon Press: New York, 1994; Vol. 12.

112. Galkin, A. G.; Kutsenko, A. S.; Bajulina, N. P.; Esipova, N. G.; Lamzin, V. S.; Mesentsev, A. V.; Shelukho, D. ;Tikhonova T. V.; Tishkov, V. I.; Ustinnikova, T. B.; Popov, V.O. *Biochim. et Biophys. Acta* 2002, *1594*, 136-149.

113. Martzen, M., R.; M Martinez, M. R.; McCraith, S. M.; Spinelli, S. L.; Torres, F. M.; Fields, S.; Grayhack, E. J.; Phizicky, E. M. *Science* 1999**,** *286,* 1153-1155.

114. Hudson, J. R.; Dawson, E. P.; Rushing, K. L.; Jackson, C. H.; Lockshon, D.; Conover, D.; Lanciault, Ch.; Harris,J.R.; Simmons, S. J.; Rothstein, R.; Fields S. *Genome Research* 1997, *7*, 1169-1173.

115. Mitchell, D.; Marhall, K.; Deschenes, R. *Yeast* 1993, *9*, 715-723.

116. Smith, D. B.; *Molecular and Biochemical Parasitology* 1988, *27*, 249-256.

117. Macreadie, I.G., Horaitis, O.; Vrkuylen, A. J.; Savin, K. W. *Gene* 1991, *104,* 107-111.

118. Macreadie, I. G.; Jagadish, M. N.; Azad M. N.; Vaughan, P. R. *Plasmid* 1989, *21*, 147-150.

119. Alister, C.; Castelli, L.; Macreadie, I. G.; Azad, A. *Yeast* 1994, *10,* 441-449.

120. Simson, P.C.; Vander, D. L. *Anal. Biochem*. 1977, *82*, 334-341.

121. Mannervik, B. *Adv. Enzymol*. 1985, *57*, 357-417.

122. Wong, C.-H.; Whitesides, G. M. *J. Am. Chem. Soc*. 1981, *103*, 4890.

123. Smith, D. B.; Johnson, K. S. *Gene* 1988**,** *67*, 31-40.

124. YOR388c, YMR318c, YCR105w, YJR159w.

125. Mori, K. *Tetrahedron* 1989, *45*, 3233-3298.

126. Wills, M.; Hannedouche, J. *Curr. Opin. Drug Disc. Devel.* 2002, *5*, 881-891.

127. Soai, K.; Yamanoi, T.; Hikima, H.; Oyamada, H. *J. Chem. Soc. Chem. Commun.* 1985, 138-139.

128. Marcantoni, E.; Alessandrini, S.; Malavolta, M. *J. Org. Chem.* 1999, *64*, 1986-1992.

129. Burk, M. *J. Acc. Chem. Res.* 2000, *33*, 363-372.

130. Evans, D. A.; Bartroli, J.; Shih, T. L. *J. Am. Chem. Soc.* 1981, *103,* 2127-212.

131. Senkan, S. M. *Nature* 1998, *394*, 350-353.

132. Maxwell, I. E.; van den Brink, P.; Downing, R. S.; Sijpkes, A. H.; Gomez, S.; Maschmeyer, T. *Top. Catal.* 2003, *24*, 125-135.

133. Kim, J.-Y.; Choi, G.-S.; Jung, I.-S.; Ryu, Y.-W.; Kim, G.-J. *Protein Eng.* 2003, *16*, 357-364.

134. Baraldi, P. T.; Zarbin, P. H. G.; Vieira, P. C.; Correa, A. G. *Tetrahedron: Asymmetry* 2002, *13*, 621-624.

135. Rodriguez, S.; Kayser, M. M.; Stewart, J. D. *J. Am. Chem. Soc.* 2001, *123*, 1547-1555.

136. Sih, C. J.; Zhou, B.-N.; Gopalin, A. S.; Shieh, W.-R.; VanMiddlesworth, F. *Sel., Goal. Synth. Effic., Proc. Workshop Conf. Hoechst. 14th* 1983, *184*, 251-261.

137. Nakamura, K.; Kondo, S.-i.; Kawai, Y.; Ohno, A. *Bull. Chem. Soc. Jpn.* 1993, *66*, 2738-2743.

138. Beck, G.; Jendralla, H.; Kesseeler, K. *Synthesis* 1995, 1014-1017.

139. Kaluzna, I.A.; Matsuda, T.; Sewall, A. K.; Stewart, J.D. *J. Am. Chem. Soc.* accepted.

140. Wolberg, M.; Kaluzna, I A.; Muller, M.; Stewart, J. D. *Tetrahedron: Asymmetry* accepted.

141. Genet, J. P.; Cano de Andrade, M. C.; Ratovelomanana-Vidal, V.; *Tetrahedron Lett.* 1995, *36*, 2063.

142. Murakami, N.; Mochizuki, M. Eur. Pat. Appl. 1990, EP 0391, 345, A1; C.A. 1991, *115*, P: 69949b.

143. Cabon, O.; Buisson, D.; Larcheveque, M.; Azerad, R. *Tetrahedron: Asymmetry* 1995, *6*, 2199-2210.

144. Cabon, O.; Larcheveque, M.; Buisson, D.; Azerad, R. *Tetrahedron: Asymmetry* 1995, *6*, 2111.

145. Akita, H.; Matsukura, H.; Oishi, T. *Tetrahedron Lett.* 1986, *27*, 5397-5400.

146. Hamdani, M.; De Jeso, B.; Deleuze, H.; Saux, A.; Maillard, B. *Tetrahedron: Asymmetry* 1991, *2*, 867-870.

147. Shibatani, T. 1991, Eur. Pat. 91103837.0.

148. Hamdani, M.; De Jeso, B.; Deleuze, H.; Saux, A.; Maillard, B. *Tetraheron: Asymmetry* 1993, *4*, 1233-1236.

149. *Chem. Pharm. Bull.* 1993, *41*, 643-648.

150. The experiment was done in our laboratory, unpublished results.

151. Wani, M.C.; Taylor, H.L.; Wall, M.E.; Caggon, P.; Mcphail, A.T. *J. Am. Chem. Soc.* 1971 ,*93,* 2325.

152. Denis, J.N.; Green, A.E.; Guenard, D.; Gueritte,V.F.; Mangatal, L.; Potier, P. *J. Am. Chem. Soc.* 1988 ,*110* ,5917.

153. Bunnage, M.E.; Davies, S.G.; Goodwin, C.J. *J. Chem. Soc., Perkin Trans.1* 1994 ,2385.

154. Kearns, J.; Kayser, M. M. *Tetrahedron Lett.* 1994 , *35*, 2845.

155. Kayser, M. M.; Mihovilovic, M. D.; Kearns, J.; Feicht,A.; Stewart, J. D. *J. Org. Chem.* 1999 , *64*, 6603.

156. Welch, J. T. *Tetrahedron* 1987, *43*, 3123-3197.

157. Filler, R.; Kobayashi, Y., *Biomedical Aspects of Fluorine Chemistry*; Eds.; Kodansha and Elsevier Biomedical: Tokoyo, 1982.

158. Bravo, P.; Resnati, G. *Tetrahedron: Asymmetry* 1990, *1*, 661-692.

159. Welch, J. T.; Eswarakrishnan, S. In *Fluorine-Containing Molecules; Structure, Reactivity, Synthesis, and Applications*; Liebman, J. F., Greenberg, A., Dolbier, W. R., Jr., Eds.; VCH: New York, 1988; Vol. 7, pp 123-147, Chapter 7.

160. Brandange, S.; Dahlman, O.; Morch, L. *J. Am. Chem. Soc.* 1981, *103*, 4452-4458.

161. Stabel, A.; Dasaradhi, L.; O'Hagan, D.; Rabe, J. P. *Langmuir* 1995, *11*, 1427.

162. O'Hagan, D.; Rzepa, H. S. *Chem. Comm.* 1997, *7*, 645-652.

163. Mann, J. *Chem. Soc. Rev.* 1987, *16*, 318.

164. Walsh, C. *Tetrahedron* 1982, *38*, 871.

165. Walsh, C. *Adv. Enzymol.* 1982, *55*, 197.

166. Walton, A. Z.; Stewart, J. D. *Biotechnol. Prog.* 2004, *20*, 403-411.

167. Katz, M.; Frejd, T.; Hahn-Hagerdal, B.; Gorwa-Grauslund M. F. *Biotechnology Bioengineering* 2003, *84*, 573-582.

168. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning. A Laboratory Manual;* Cold Spring Harbor: Cold Spring Harbor, 1989.

169. Guthrie, C.; Fink, G. R. *Methods in Enzymology* 1991, *194.*

170. Wach, A.; Pick, H.; Philippsen, P. In *Genetics of Yeast. The Practical Approach.*; Johnston, J. R., Ed.; IRL Press: New York, 1994.

171. Bradford, M. M. *Anal. Biochem.* 1976, *72,* 248-254.

172. Kim, D. Y.; Park, E. J. *Organic Letters* 2002, *4*, 545-547.

173. Hofle, G.; Steglich, W.; Vorbruggen, H. *Angew. Chem. Inter. Ed. Engl.* 1978, *17*, 569.

BIOGRAPHICAL SKETCH

Iwona Agata Kaluzna was born in Krosno Odrzanskie, Poland, on August 3, 1974. She graduated with a master's Degree in Chemistry from Adam Mickiewicz University Poznan, Poland, in May of 1999. She received her master degree in supramolecular chemistry, where she studied kinetics of complexation reactions between lariat ethers and iron II and III atoms under supervision of Prof. Grzegorz Schroeder. In August of 1999, she joined the chemistry research group of Dr. Jon D. Stewart at the University of Florida to continue her graduate studies. Upon completion of her studies, she will be working for the biotechnology company, BioCatalytics.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jon D. Stewart, Chair
Associate Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Nicole Horenstein
Associate Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Nigel G. J. Richards
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jonathan E. Enholm
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Henry V. Baker
Professor and Chairman Department of
Molecular Genetics and Microbiology

This dissertation was submitted to the Graduate Faculty of the Department of Chemistry in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 2004

_____

Dean, Graduate School